COMPOSITIONS AND METHODS FOR DIAGNOSING AND TREATING CONDITIONS, DISORDERS, OR DISEASES INVOLVING CELL DEATH

TABLE OF CONTENTS

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1 INTRODUCTION

The present invention relates to compositions and methods for the treatment and diagnosis of conditions, disorders, or diseases involving cell death, including, but not limited to, neurological disorders such as stroke. Nucleic acids are described herein which, when introduced into a cell either predisposed to undergo cell death or in the process of undergoing cell death, prevent, delay, or rescue the cell from death relative to a corresponding cell into which no exogenous nucleic acids have been introduced Such nucleic acids are referred to as "protective sequences". Protective sequences or their products are identified by their ability to prevent, delay, or rescue a cell, cells, tissues, organs, or organisms from dying. Protective sequences or their products are also identified via their ability to interact with other genes or gene products involved in conditions or disorders involving cell death.

The invention further includes recombinant DNA molecules and cloning vectors comprising protective sequences, and host cells and host organisms engineered to contain such DNA molecules and cloning vectors. The present invention further relates to protective sequence products and to antibodies directed against such protective sequence products.

The protective sequences identified, their products, or antibodies may be used diagnostically, prophylactically, therapeutically or as targets for therapeutic intervention. In this regard, the present invention provides methods for the identification and prophylactic or therapeutic use of compounds in the treatment and diagnosis of conditions, disorders, or diseases involving cell death. Additionally, methods are provided for the diagnostic monitoring of patients undergoing clinical evaluation for the treatment of conditions or disorders involving cell death, for monitoring the efficacy of compounds in clinical trials and for identifying subjects who may be predisposed to such conditions, disorders, or diseases involving cell death.

2 BACKGROUND OF THE INVENTION

2.1 Mechanisms which Lead to Cell Death

It is widely recognized that at least two distinct cell death mechanisms exist for mammalian cells. These two mechanisms are necrosis and apoptosis, and are significant components of numerous conditions, disorders and disease states.

Necrosis plays an important physiologic role in signaling the presence of certain conditions. When cells die as a result of necrosis, the dying cells release substances that activate the body's immune response in a local, and in some cases widespread, reaction to the necrosis-inducing condition. This response is important in, for example, bacterial infection.

Experimental evidence in a wide variety of cells throughout the body has revealed that every cell can initiate a program of self-destruction, called apoptosis. This program can be initiated by a wide variety of natural and unnatural events. There are at least four distinct pathways for executing this program of cell death, and it is virtually certain that dozens, if not hundreds, of different intracellular biochemical cascades interact with each pathway. It is equally likely that certain cell types, such as cells in the heart or neurons, will utilize specialized signaling pathways that are not generally represented elsewhere in the body. However, since cell death is neither always necessary nor desired, it would be desirable to manipulate the manner in which cells start their death process. In some circumstances, preventing, delaying, or rescuing cells from death would either alleviate the disease or allow more time for definitive treatment to be administered to the patient. An example of this situation is brain cell death caused by ischemic stroke: preventing, delaying, or rescuing cells from death until the blood supply to the brain could be restored would greatly reduce, if not eliminate, the possibility of a person's death and/or long-term disability from stroke (Lee JM, et al. *Nature* 1999, 399(supp): A7-A14; Tarkowski E, et al. *Stroke* 1999, 30(2): 321-7; Pulera MR, et al. *Stroke* 1998, 29(12): 2622-30). In still other circumstances, the failure of cells to die may itself lead to disease such as cancer (Hetts SW. *JAMA* 1998, 297(4): 300-7).

Cell death plays an important role in the normal function of mammalian organisms. While it may seem counterintuitive for cells to have death as a normal part of their life cycle, controlled and physiologically appropriate cell death is important in regulating both the absolute and relative numbers of cells of a specific type. (Hetts SW. *JAMA* 1998, 297(4): 300-7; Garcia I, et al. *Science* 1992, 258(5080): 302-4). When the mechanism of apoptosis does not function properly and normal cell death does not occur, the resulting disease is characterized by unregulated cellular proliferation, as occurs in a neoplastic disease or an autoimmune disease (Hetts SW. *JAMA* 1998, 297(4): 300-7; Yachida M, et al. *Clin Exp Immunol* 1999, 116(1): 140-5).

One method for regulating cell death involves manipulating the threshold at which the process of cell death begins. This threshold varies significantly by cell type, tissue type, the type of injury or insult suffered by the cell, cellular maturity, and the physiologic conditions in the cell's environment (Steller H., *Science* 1995, 267(5203): 1445-9). Although it is probable that certain cellular injuries or insults irrevocably induce death, lesser injuries or insults may begin the dying process without inducing irreversible cell death. What constitutes a lesser injury or insult may vary tremendously with changes in the factors influencing that cell's death threshold. The ability to alter a cell's threshold for responding to an injury or insult, that is, to either promote or discourage cell death, would be a desirable goal for the treatment of conditions involving cell death. The ability to better control cell death, by either discouraging or promoting the mechanisms of cell death, would be an important invention for ameliorating disease (US Patents 5,925,640; 5,786,173; 5,858,715; 5,856,171).

Recent evidence suggests that the mechanisms of cellular death may be more complex than the two discrete pathways of apoptosis and necrosis. Examples of this evidence may be found in the central nervous system (CNS). In the complex CNS cellular environment, both necrosis and apoptosis are observed with commonly studied conditions, disorders, or diseases such as focal ischemia, global ischemia, toxic insults, prolonged seizures, excitotoxicity, and traumatic brain injury. In some reports, both apoptosis and necrosis have been described (Choi WS, et al. *J Neurosci Res* 1999, 57(1): 86-94; Li Y, et al. *J Neurol Sci* 1998, 156(2): 119-32; Lee J-M, et al. *Nature* 1999, 399(supp): A8-A14; Baumgartner WA, et al. *Ann Thorac Surg* 1999, 67(6): 1871-3; Fujikawa DG, et al. *Eur J Neurosci* 1999, 11(5): 1605-14; Gwag BJ, et al *Neuroscience* 1999, 90(4): 1339-48; Mitchell IJ, et al. 1998, 84(2): 489-501; Nakashima K, et al. *J Neurotrauma* 1999, 16(2): 143-51; Ginsburg, MD *Cerebrovascular Disease: Pathophysiology, Diagnosis, and Management* 1998 Ch 42; Rink AD, et al. *Soc Neurosci Abstr* 1994, 20:250(Abstract)). Similar observations also occurred with brain tumor cells. (Maurer BJ, et al. *J Natl Cancer Inst* 1999, 91(13): 1138-46) Other investigators found that neurons die by either apoptosis or necrosis under different environmental conditions (Taylor DL, et al. *Brain Pathol* 1999, 9(1): 93-117). There also are reports of a unique type of neuronal cell death following stroke. This new type of cell death has features common to both necrosis and apoptosis (Fukuda T, et al. *Neurosci Res* 1999, 33(1): 49-55). Other investigators believe that neuronal cell death is best represented by a continuum between apoptosis and necrosis, possibly mediated by calcium

levels (Lee J-M, et al.1999, 399(supp): A7-A14), or a combination of direct ischemic damage followed by indirect damage from excitotoxicity and loss of interneuronal connections (Martin LJ, et al. *Brian Res Bull* 1998, 46(4): 281-309). Further complicating the picture of neuronal cell death is the observation that the death of one or more neurons in one region of the brain can induce the death of neurons in other brain regions. This phenomenon has been observed with stroke as described above (Martin LJ, et al. *Brain Res Bull* 1998, 46(4): 281-309) as well as neuronal cell death induced by the withdrawal of growth factors (Ryu BR, et al. *J Neurobiol* 1999, 39(4): 536-46). Given the complex nature of actions and interactions among the many physiologic and molecular forces in brain tissue, and the different abilities of many substances acting either alone or in combination to induce cellular injury or death, it is difficult to determine with any degree of certainty if a nerve cell death process is due to apoptosis or necrosis (Graham DI, *Greenfield's Neuropathology* Ch 3 1997).

Despite the challenges in classifying the mechanism of cellular death, there is broad agreement that most, if not all, cells share common features in their death mechanisms (see, *e.g.*, Lee J.M., et al., *Nature* 1999, 399 (supp): A7-A14).

2.2 Selected Factors and Conditions which Inhibit Cell Death Mechanisms

Several factors have been reported to inhibit the cell death pathway. One of the best-known factors is the gene product *bcl-2* (Adams JM, et al. *Science* 1998, 281(5381): 1322-6; Vaux DL, et al. *Proc Natl Acad Sci* 1993, 90(3): 786-9; US Patent 5,856,171 and references cited therein). Expression of *bcl-2* is believed to regulate apoptotic death in neurons, kidney, heart, liver, blood and skin cells under experimental conditions. In addition to regulating death by apoptosis, *bcl-2* is believed to regulate death caused by non-apoptotic mechanisms. Factors related to *bcl-2* have been shown to be over-expressed in cancer and autoimmune conditions, disorders, or diseases (US Patent 5,856,171 and references cited therein). Other related factors acting on the same pathway as *bcl-2* also delay or prevent cell death.

In the brain, several factors have been shown to influence the cell death pathway. In excitotoxic injury to neurons, it was shown that lithium or *bcl-2* each individually protected neurons against cell death (Nonaka S, et al. *Proc Natl Acad Sci* 1998, 95(5): 2642-7; Behl C, et al. *Biochem Biophys Res Commun* 1993, 197(2): 949-56). During ischemic injury to neurons, it was shown that nerve growth factor (NGF) and *bcl-2*

individually offered protection against neuronal death (Guegan C, et al. *Neurobiol Dis* 1999, 6(3): 180-9; Linnik MD, et al. *Stroke* 1995, 26(9): 1670-4).

Factors acting to prevent cell death do not act solely in the brain. In the heart, increased tolerance to non-lethal ischemic injury was associated with an increased expression of the *bcl-2* gene, suggesting that *bcl-2* was involved in protecting the cardiac muscle cells against ischemic injury (Maulik N, et al. *Ann NY Acad Sci* 1999, 874:401-11). This same study demonstrated that lower levels of *bcl-2* expression were associated with higher rates of cardiac cell death. A similar result was found for mechanical injury to heart papillary muscle cells.

Recently, it has been demonstrated that *bcl-2* prevented cell death in a brain ischemia model (Guegan C, et al. *Neurobiol Dis* 1999, 6(3): 180-9; Linnik MD, et al. *Stroke* 1995, 26(9): 1670-4). It was shown that the activity of *bcl-2* to prevent neuronal death was consistently demonstrated across several different physiologic insults. It also has been demonstrated that the distinction between apoptotic death and necrotic death is open to question, so the possibility exists that *bcl-2* can prevent or delay the necrotic cell death pathway, the apoptotic cell death pathway or perhaps an as yet undemonstrated cell death pathway.

Preventing cell death is an important medical goal. Several types of mammalian cells, most notably neurons and cardiac muscle cells, have limited if any capacity to regenerate. Preventing the death of these cells from conditions such as heart attack, stroke, shock, infection, cancer, Alzheimer's disease or traumatic injury, to name a few, would be an important medical advance as the heart and brain cannot grow sufficient cells to replace those cells lost to disease or infection.

In addition to preventing cell death, delaying and/or rescuing cells from programmed cell death is also an important medical goal. In many pathological conditions where there is an expectation that the disease will be successfully treated, such as many types of infection, hypoxia, ischemia or metabolic disturbances, delaying cell death would allow the pathological condition to be treated without permanent damage to the cells. In other words, the cells may be put into a suspended state from which they could successfully be rescued and emerge with their normal function intact.

3 SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification and characterization of protective sequences and to compositions and methods for the treatment and diagnosis of conditions, disorders, or diseases involving cell death. Protective sequences refer to nucleic acid molecules comprising nucleic acid sequences which, when introduced into a cell either predisposed to undergo cell death or in the process of undergoing cell death, prevent, delay, or rescue the cell from death relative to a corresponding cell into which no exogenous nucleic acids have been introduced. For example, protective sequences may act to prevent, delay, ameliorate, inhibit, reduce, or rescue neuronal cell death (*e.g.* apoptosis, necrosis and related cellular events). The invention further relates to the discovery, identification and characterization of gene products encoded by such nucleic acid molecules, or by degenerate, *e.g.*, allelic or homologous, variants thereof. Protective sequences also can be regulatory nucleic acids. Protective sequences further can be both coding sequences and regulatory sequences.

The invention further relates to target sequences. Target sequences include, but are not limited to, upstream and downstream regulatory sequences, upstream and downstream complete or partial gene or gene product sequences, antibodies, antisense molecules or sequences, ribozyme molecules, and other inhibitors or modulators directed against such protective sequences and protective sequence products.

Protective sequences and protective sequence products can be utilized prophylactically and/or therapeutically to prevent, delay ameliorate, inhibit, reduce, or rescue conditions of cell death or symptoms of conditions, disorders, or diseases involving cell death. The modulation of the expression of protective sequences, *e.g.*, endogenous protective sequences, and/or the activity of the protective sequence products, *e.g.*, endogenous protective sequence products, can also be utilized prophylactically or therapeutically to prevent, delay, ameliorate, inhibit, reduce, or rescue conditions of cell death or symptoms of conditions, disorders, or diseases involving cell death. Further, protective sequences and protective sequence products can be used to diagnose individuals exhibiting or predisposed to such conditions, disorders, or diseases involving cell death.

The compositions of the present invention include, in particular, nucleic acid molecules which comprise the following sequences: (a) nucleic acids of protective sequences, as well as allelic variants, homologs, mutants and fragments thereof; (b) nucleic acids which encode protective sequence products; (c) nucleic acids which encode protective

sequence regulatory elements; (d) nucleic acids which encode fusion proteins comprising protective sequence products or one or more protective sequence product domains fused to a heterologous polypeptide; (e) nucleic acids which encode fusion proteins comprising protective sequence regulatory elements fused to a heterologous polypeptide; (f) nucleic acids which hybridize to the above described sequences under highly stringent or moderately stringent conditions, including, but not limited to, human homologs; and (g) complementary (*e.g.*, antisense) nucleic acids of the sequences described in (a) through (f), above. The nucleic acid molecules of the invention include, but are not limited to, cDNA, genomic DNA and RNA sequences.

The present invention also encompasses expression gene products of the protective sequences listed above; *i.e.*, proteins and/or polypeptides that are encoded by the above protective sequences.

Mimics, agonists and antagonists of the protective sequences, protective sequence products, genes, gene products, or their regulatory elements are also included in the present invention. Such mimics, agonists and antagonists will include, for example, small molecules, large molecules (*e.g.*, protective sequence product fragments or protective sequence product ligands) and antibodies directed against a protective sequence product. Mimics, agonists and antagonists of the invention also include nucleic acids, such as antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs, which can be used to modulate, inhibit or enhance expression of a protective sequence.

The present invention further encompasses cloning and expression vectors, which may include, but are not limited to, bacterial, fungal, insect, plant, and mammalian vectors, which contain the protective nucleic acid sequences of the invention, which can be used as probes or to express those protective nucleic acid sequences, protective sequence products, genes and/or gene products in host cells or organisms. The present invention also relates to cells that have been transformed, transfected, or infected with such vectors, and to cells engineered to contain or express the protective nucleic acid sequences, protective sequence products, genes, gene products, and/or regulatory elements of the invention. Further, non-human host organisms which have been transformed, transfected, or infected with these protective nucleic acid sequences, or their regulatory elements, are also encompassed in the present invention. Host organisms of the invention include organisms transformed, transfected, or infected with the cloning vectors described above, including, but not limited to, non-human transgenic animals, and particularly transgenic non-human

mammals which have been engineered to express a protective sequence, protective sequence product, gene, gene product, or regulatory element of the invention, or "knock-outs" which have been engineered to not express the protective sequence, protective sequence product, gene, gene product, or regulatory element of the invention.

The transgenic animals of the invention include animals which express a mutant variant or polymorphism of a protective sequence, protective sequence product, gene, gene product, or regulatory element, particularly a mutant variant or polymorphism of a protective sequence, protective sequence product, gene, gene product, or regulatory element which is associated with a condition, disorder, or disease involving cell death. The transgenic animals of the invention further include those that express a protective sequence transgene at higher or lower levels than normal. The transgenic animals of the invention further include those which express the protective sequence, protective sequence product, gene, gene product, or regulatory element in all their cells, "mosaic" animals which express the protective sequence, protective sequence product, gene, gene product, or regulatory element in only some of their cells, and those in which the protective sequence, protective sequence product, gene, gene product, or regulatory element is selectively introduced into and expressed in a specific cell type(s). The transgenic animals of the invention also include "knock-out" animals. Knock-out animals comprise animals that have been engineered to no longer express the protective sequence, protective sequence product, gene, gene product, or regulatory element.

The present invention also relates to methods and compositions for the diagnosis of conditions, disorders, or diseases involving cell death, as well as for the identification of subjects susceptible to such conditions, disorders, or diseases. Such methods comprise, for example, measuring expression of the protective sequence, protective sequence product, gene, gene product, or regulatory element in a patient sample, or detecting a mutation in the protective sequence, protective sequence product, gene, gene product, or regulatory element in the genome of a mammal, including a human, suspected of exhibiting such a condition, disorder, or disease. The protective nucleic acid molecules of the invention can be used also as diagnostic hybridization probes, or as primers for diagnostic PCR analysis to identify protective sequences, protective sequence products, genes, gene products, or regulatory element mutations, allelic variations or regulatory defects, such as defects in the expression of the protective sequence, protective sequence product, gene, gene product, or regulatory element. Such diagnostic PCR analyses can be used to diagnose individuals with a

condition, disorder, or disease involving cell death associated with a particular protective sequence, protective sequence product, gene, gene product, or regulatory element mutation, allelic variation or regulatory defect. Such diagnostic PCR analyses can be used also to identify individuals susceptible to such conditions, disorders, or diseases involving cell death.

Methods and compositions, including pharmaceutical compositions, for the treatment of conditions, disorders, or diseases involving cell death also are included in the invention. Such methods and compositions can increase, decrease or otherwise modulate the level of protective sequences, protective sequence products, genes, gene products, or their regulatory elements in a patient in need of such treatment. Such methods and compositions can also modulate the level of protective sequence expression (*e.g.*, endogenous protective sequence expression) and/or the level of activity of a protective sequence product, (*e.g.*, endogenous protective sequence product). Further, since the protective sequence or protective sequence product need not normally be involved in such conditions, disorders, or diseases, such methods include, for example, modulating the expression of the protective sequence and/or the activity of the protective sequence product for the treatment of conditions, disorders, or diseases involving cell death which are normally mediated by some other gene.

In one embodiment, such methods and compositions are utilized for the treatment of the types of conditions, disorders, or diseases, which can be prevented, delayed or rescued from cell death and include, but are not limited to, those associated with the central nervous system including neurological and psychiatric conditions, disorders, or diseases; those of the peripheral nervous system; conditions, disorders, or diseases caused by physical injury; conditions, disorders, or diseases of the blood vessels or heart; conditions, disorders, or diseases of the respiratory system; neoplastic conditions, disorders, or diseases; conditions, disorders, or diseases of blood cells; conditions, disorders, or diseases of the gastrointestinal tract; conditions, disorders, or diseases of the liver; conditions, disorders, or diseases of the pancreas; conditions, disorders, or diseases of the kidney; conditions, disorders, or diseases of the ureters, urethra or bladder; conditions, disorders, or diseases of the male genital system; conditions, disorders, or diseases of the female genital tract; conditions, disorders, or diseases of the breast; conditions, disorders, or diseases of the endocrine system; conditions, disorders, or diseases of the thymus or pineal gland; conditions, disorders, or diseases of the skin or mucosa; conditions, disorders, or diseases of the musculoskeletal system; conditions, disorders, or diseases causing a fluid or hemodynamic

derangement; inherited conditions, disorders, or diseases; conditions, disorders, or diseases of the immune system or spleen; conditions, disorders, or diseases caused by a nutritional disease; and conditions, disorders, or diseases typically occurring in infancy or childhood, as described in Section 5.4.1.1. below.

In yet another embodiment, the methods and compositions of the invention are utilized for the prevention, or delay, of cell death in the event of one or more infections which may be caused by bacteria; viruses; members of the family rickettsiae or chlamydia; fungi, yeast, hyphae or pseudohyphae; prions; protozoans; or metazoans.

In a further embodiment, the compounds and methods of the invention can be used to treat infections or conditions, disorders, or diseases which cause cell death in organ systems including, but not limited to, blood vessels, heart, red blood cells, white blood cells, lymph nodes, spleen, respiratory system, oral cavity, gastrointestinal tract, liver and biliary tract, pancreas, kidney, lower urinary tract, upper urinary tract and bladder, male sexual organs and genitalia, female sexual organs and genitalia, breast, thyroid gland, adrenal gland, parathyroid gland, skin, musculoskeletal system, bone marrow or bones.

In another embodiment, the compounds and methods of the invention can be used to treat further physiological impacts on organs caused by the infections which induce cell death including, but not limited to, fever equal to or greater than 101.5 degrees Fahrenheit, a decrease or increase in pulse rate by more than 20 beats per minute, a decrease or increase in supine systolic blood pressure by more than 30 millimeters of mercury, an increase or decrease in respiratory rate by more than 8 breaths per minute, an increase or decrease in blood pH by more than 0.10 pH units, an increase or decrease in one or more serum electrolytes outside of the clinical laboratory's usual reference range, an increase or decrease in the partial pressure of arterial oxygen or carbon dioxide outside of the clinical laboratory's usual reference range, an increase or decrease in white or red blood cells outside of the laboratory's usual reference range, an acute confusional state such as delirium where delirium is defined by the American Psychiatric Association's DSM-IV Manual or a diminished level of consciousness or attention.

In another embodiment, the compounds and methods of the invention can be used to promote cell death. These compounds could be useful for treating and/or ameliorating conditions caused by, for example, cancer and autoimmune diseases, both of which are manifested by an uncontrolled growth of cells.

The invention still further relates to methods for identifying compounds which

modulate the expression of a protective sequence and/or the synthesis or activity of a protective sequence product. Such compounds include therapeutic compounds which can be used as pharmaceutical compositions to reduce or eliminate the symptoms of conditions, disorders, or diseases involving cell death. Cellular and non-cellular assays are described which can be used to identify compounds which interact with a protective sequence, protective sequence product, gene, gene product, and/or regulatory element, *e.g.*, modulate the activity of a protective sequence and/or bind to a protective sequence product. Such cell-based assays of the invention utilize cells, cell lines, or engineered cells or cell lines that express the protective sequence, protective sequence product, gene, gene product, and/or regulatory element.

In one embodiment, such methods comprise contacting a compound to a cell which expresses a protective sequence, protective sequence product, gene, gene product, and/or regulatory element, measuring the level of protective sequence expression, gene product expression or gene product activity, and comparing this level to the level of protective sequence expression, gene product expression or gene product activity produced by the cell in the absence of the compound, such that if the level obtained in the presence of the compound differs from that obtained in its absence, a compound which modulates the expression of the protective sequence and/or the synthesis or activity of protective sequence products has been identified.

In an alternative embodiment, such methods comprise administering a compound to a host, *e.g.*, a transgenic animal which expresses a protective sequence transgene or a mutant protective sequence transgene, and measuring the level of protective sequence expression, gene product expression or gene product activity. The measured level is compared to the level of protective sequence expression, gene product expression or gene product activity in a host which is not exposed to the compound, such that if the level obtained when the host is exposed to the compound differs from that obtained when the host is not exposed to the compound, a compound which modulates the expression of the protective sequence and/or the synthesis or activity of protective sequence products, and/or the symptoms of conditions, disorders, or diseases involving cell death, has been identified.

3.1 Definitions

"Protective sequence", as used herein, refers to nucleic acid molecules comprising nucleic acid sequences which, when introduced into a cell predisposed to either

undergo cell death or in the process of undergoing cell death, prevent, delay, or rescue the cell from death relative to a corresponding cell into which no exogenous protective nucleic acids have been introduced. In one embodiment, a protective sequence encodes a protective sequence product. In another embodiment, protective sequences are any transcriptional products of the sequences disclosed herein. In another embodiment, protective sequences comprise regulatory elements of the sequences disclosed herein which modulate the expression of a nucleic acid within a cell. For example, protective sequences, their products, or their regulatory elements may act to prevent, delay, or rescue a cell, cells, tissues, organs, or organisms from dying. Compounds which modulate protective sequence expression or activity of the protective sequence product can be used in the treatment of conditions, disorders or diseases associated with cell death processes. It is to be understood that the protective sequences described above can act to ameliorate or delay symptoms related to cell death. Although the protective sequences may be involved directly in such cell death related conditions or disorders, in certain cases, the protective sequences will not normally be involved in such conditions or disorders, but will be effective for the treatment and/or prevention of such disorders. In these cases, modulation of the expression of the protective sequence and/or the activity of the protective sequence product will be useful for the treatment of conditions, disorders, or diseases involving cell death which are normally mediated by some other gene.

"Cell death", as used herein, refers to any mechanism and/or pathway whereby a cell undergoes a series of events which ultimately would lead to the death of the cell. For example, cell death may be caused by various processes including, but not limited to, apoptosis or programmed cell death, necrosis, or an as yet unidentified cell death pathway. Cell death may be induced in individual cells as a consequence of numerous internal and external stimuli including, but not limited to, genetic predisposition, toxic chemicals or processes, heat, cold, rapid environmental changes, radiation, viruses, prions, bacteria, disruption of nutrient balance, or exposure to bi-products and signaling from other cells undergoing cell death. The protective sequences disclosed herein, when introduced into a cell (*e.g.* a neuronal cell) which has undergone an event that would ultimately lead to cell death (*e.g.* ischemia), are capable of rescuing the cell from cell death. Moreover, when a protective sequence, in combination with a reporter gene (*e.g.* green fluorescent protein), is introduced into a cell which has undergone an event that would ultimately lead to cell death, expression

of the reporter gene is an indication that the protective sequence is capable of rescuing the cell from cell death.

4 BRIEF DESCRIPTION OF THE FIGURES

Figures 1(A-J). Protective nucleic acids. See Table 1 for the identity, the sequence identifier number, the length in base pairs and the Accession Number for each of the sequences shown in these figures.

Figure 2. Restriction map and diagram of plasmid pCMV•SPORT2. This plasmid was used as the cloning vector for the protective sequences. Each clone was ligated into the *SalI-NotI* restriction sites of the plasmid.

Figures 3(A- N). Protected Cortical Neurons Visualized by Detection of EGFP Expressing Cells. Figures 3A and 3B represent non-stroked, positive control samples. Figure 3C represents a positive control, stroked sample using Bcl-2. Figure 3D represents a stroked, negative control sample. Figure 3E represents a stroked sample protected by CNI-00711. Figure 3F represents a stroked sample protected by CNI-00712. Figure 3G represents a stroked sample protected by CNI-00714. Figure 3H represents a stroked sample protected by CNI-00715. Figure 3I represents a stroked sample protected by CNI-00716. Figure 3J represents a stroked sample protected by CNI-00717. Figure 3K represents a stroked sample protected by CNI-00720. Figure 3L represents a stroked sample protected by CNI-00721. Figure 3M represents a stroked sample protected by CNI-00723. Figure 3N represents a stroked sample protected by CNI-00724.

Figures 4(A-L). Open Reading Frames for CNI-00711. This Figure depicts the twelve (12) potential ORFs for CNI-00711. Also shown are the nucleotide sequences which encode the ORFs.

Figures 5(A-X). Open Reading Frames for CNI-00712. This Figure depicts the 24 potential ORFs for CNI-00712. Also shown are the nucleotide sequences which encode the ORFs.

Figures 6(A-AD). Open Reading Frames for CNI-00714. This Figure depicts the 30 potential ORFs for CNI-00714. Also shown are the nucleotide sequences which

encode the ORFs.

Figures 7(A-H). Open Reading Frames for CNI-00715. This Figure depicts the eight (8) potential ORFs for CNI-00715. Also shown are the nucleotide sequences which encode the ORFs.

Figures 8(A-O). Open Reading Frames for CNI-00716. This Figure depicts the fifteen (15) potential ORFs for CNI-00716. Also shown are the nucleotide sequences which encode the ORFs.

Figures 9(A-AL). Open Reading Frames for CNI-00717. This Figure depicts the 38 potential ORFs for CNI-00717. Also shown are the nucleotide sequences which encode the ORFs.

Figures 10(A-O). Open Reading Frames for CNI-00720. This Figure depicts the fifteen (15) potential ORFs for CNI-00720. Also shown are the nucleotide sequences which encode the ORFs.

Figures 11(A-AG). Open Reading Frames for CNI-00721. This Figure depicts the 33 potential ORFs for CNI-00721. Also shown are the nucleotide sequences which encode the ORFs.

Figures 12(A-AY). Open Reading Frames for CNI-00723. This Figure depicts the 51 potential ORFs for CNI-00723. Also shown are the nucleotide sequences which encode the ORFs.

Figures 13. Open Reading Frame for CNI-00724. This Figure depicts the single potential ORF for CNI-00724. Also shown is the nucleotide sequence which encodes the ORF.

DETAILED DESCRIPTION OF THE INVENTION

Protective sequences of the invention are described herein. Also described are recombinant, cloned and degenerate variants, homologs, orthologs, mutants and fragments

thereof. The compositions of the invention further include protective sequence products (*e.g.* proteins or RNA) which are encoded or produced by the nucleic acid molecules of the invention, and the modulation of protective sequence expression and/or gene product activity in the treatment of conditions, disorders, or diseases involving cell death. Further, antibodies directed against the protective sequence products, or conserved variants or fragments thereof, and viral-, cell-, plant-, and animal-based models by which the protective sequences may be further characterized and utilized are also discussed in this section.

5.1 The Protective Sequences

The protective sequences of the invention are described in this section. Specifically, these protective sequences have been shown to prevent, delay, or rescue cell death in a cell predisposed for undergoing cell death, whether the pathway that leads to the cell death involves apoptosis, necrosis or an as yet undefined pathway. The protective sequences, their SEQ ID NOS and additional information related to the protective sequences are listed below, in Table 1.

The protective sequences listed in Table 1 may be obtained using cloning methods well known to those skilled in the art, including but not limited to the use of appropriate probes to detect the protective sequences within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by reference herein in its entirety). Probes for the novel sequences reported herein may be obtained directly from CNI-NPP1-CP10, which represents a composite deposit containing the isolated clones, which was deposited with the NRRL as Accession No. B-30231. Alternatively, oligonucleotide probes for the novel protective sequences may be synthesized based on the DNA sequences disclosed herein.

TABLE 1

PROTECTIVE SEQUENCES

Protective sequence	SEQ ID NO:	Figure No.	Length (bp) (NotI-SalI fragment)
CNI-00711	*1*	*1A*	*852*
CNI-00712	*26*	*1B*	*1096*
CNI-00714	*75*	*1C*	*1825*
CNI-00715	*136*	*1D*	*542*
CNI-00716	*153*	*1E*	*771*
CNI-00717	*184*	*1F*	*1669*
CNI-00720	*261*	*1G*	*1182*
CNI-00721	*292*	*1H*	*1965*
CNI-00723	*359*	*1I*	*2702*
CNI-00724	*462*	*1J*	*979*

The isolated protective nucleic acid molecules of the invention include, in particular, nucleic acid molecules which comprise the following sequences: (a) nucleic acids of protective sequences, as well as allelic variants, homologs, mutants and fragments thereof; (b) nucleic acids which encode protective sequence products and/or their regulatory elements, or fragments thereof; (c) nucleic acids which encode fusion proteins comprising protective sequence products and/or their regulatory elements, or one or more protective sequence product domains and/or their regulatory elements fused to a heterologous polypeptide; (d) nucleic acids which hybridize to the above described sequences under highly stringent or moderately stringent conditions, including, but not limited to human homologs; and (e) complementary (*e.g.*, antisense) nucleic acids of the sequences described in (a) through (d), above. The nucleic acid molecules of the invention include, but are not limited to, cDNA, genomic DNA and RNA sequences.

The nucleic acids of the invention also include nucleic acids which have at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more nucleic acid identity to the protective nucleic acids of (a)-(d) above. The nucleic acids of the invention further include nucleic acids which encode polypeptides having at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or higher amino acid sequence identity to the polypeptides encoded by the protective nucleic acids of (a)-(d).

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical overlapping positions/total # of positions x 100). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences also can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 87*:2264-2268, modified as in Karlin

and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol. 215*:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleic acids homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.25*: 3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see http://www.ncbi.nlm.nih.gov). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) *CABIOS 4*:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

The nucleic acids of the invention further include: (a) any nucleic acid which hybridizes to a nucleic acid molecule of the invention under moderately stringent conditions, *e.g.*, hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45°C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65°C, or (b) under highly stringent conditions, *e.g.*, hybridization to filter-bound nucleic acid in 6xSSC at about 45°C followed by one or more washes in 0.1xSSC/0.2% SDS at about 68°C, or under other hybridization conditions which are apparent to those of skill in the art (*see*, for example, Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at pp. 6.3.1-6.3.6 and 2.10.3). Preferably the nucleic acid molecule that hybridizes to the nucleic acid of (a) and (b), above, is one which comprises the complement of a nucleic acid molecule which encodes a protective sequence product. In a preferred embodiment, nucleic acid molecules comprising the nucleic acids of (a) and (b), above, encode protective sequence products.

Functionally equivalent protective sequence products include naturally occurring protective sequence products present in the same or different species. Functionally equivalent protective sequence products also include gene products which retain at least one of the biological activities of the protective sequence products, and/or which are recognized by and bind to antibodies (polyclonal or monoclonal) directed against the protective sequence products.

Among the nucleic acid molecules of the invention are deoxyoligonucleotides ("oligos") which hybridize under highly stringent or moderately stringent conditions to the nucleic acid molecules described above. In general, for probes between 14 and 70 nucleotides in length the melting temperature (TM) is calculated using the formula: Tm (°C)=81.5+16.6(log[monovalent cations (molar)])+0.41 (% G+C)-(500/N) where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation Tm (°C)=81.5+16.6(log[monovalent cations (molar)])+0.41(% G+C)-(0.61% formamide)-(500/N) where N is the length of the probe. In general, hybridization is carried out at about 20-25 degrees below Tm (for DNA-DNA hybrids) or 10-15 degrees below Tm (for RNA-DNA hybrids).

Exemplary highly stringent conditions may refer, *e.g.*, to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for about 14-base oligos), 48 °C (for about 17-base oligos), 55 °C (for about 20-base oligos) and 60 °C (for about 23-base oligos).

Fragments of the nucleic acid molecules can be at least 10 nucleotides in length. Fragments of the nucleic acid molecules can refer also to exons or introns, and, further, can refer to portions of coding regions that encode domains of protective sequence products.

The invention also encompasses (a) DNA vectors which contain any of the foregoing coding sequences and/or their complements (*i.e.*, antisense); (b) DNA expression vectors which contain any of the foregoing coding sequences operatively associated with a regulatory element which directs the expression of the coding sequences; and (c) genetically engineered host cells which contain such vectors or have been engineered to contain and/or express a nucleic acid sequence of the invention, *e.g.*, any of the foregoing coding sequences operatively associated with a regulatory element which directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art which drive and regulate expression. The invention further includes

fragments of any of the DNA sequences disclosed herein.

The nucleic acid molecules may encode or act as antisense molecules, useful, for example, in protective sequence regulation, and/or as hybridization probes and/or as primers in amplification reactions of protective nucleic acid sequences. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for protective sequence regulation. Still further, such molecules may be used as components of diagnostic methods whereby, for example, the presence of a particular allele involved in a condition, disorder, or disease involving cell death may be detected.

The protective nucleic acids of the invention can be readily obtained, for example, by standard sequencing and the sequences provided herein.

As will be appreciated by those skilled in the art, DNA sequence polymorphisms of a protective sequence will exist within a population of individual organisms (*e.g.*, within a human population). Such polymorphisms may exist, for example, among individuals within a population due to natural allelic variation. Such polymorphisms include ones that lead to changes in amino acid sequence. An allele is one of a group of alternative forms of a gene that occur at a given genetic locus.

As used herein, the phrase "allelic variant" refers to a nucleic acid that occurs at a given locus or to a gene product encoded by that nucleic acid. Such natural allelic variations can typically result in 1-5% variance in the nucleic acid of a given gene. Sequencing the gene of interest in a number of different individuals can identify alternative alleles. Using hybridization probes to identify the same genetic locus in a variety of individuals can readily carry this out.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising any of up to six open reading frames which may or may not encode a polypeptide of the invention. For example, the terms "gene" and "recombinant gene" refer to nucleic acid molecules encoding any of the open reading frames shown in Figures 4-13, and described in Tables 2-11, respectively. The term can further include nucleic acid molecules comprising upstream and/or exon/intron sequences and structures.

TABLE 2

OPEN READING FRAMES FOR CNI-00711

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	30 Nucleotide	48-77 of Seq. Id. No. 1	2
	9 Amino Acid		3
2	60 Nucleotide	78-137 of Seq. Id. No. 1	4
	19 Amino Acid		5
3	12 Nucleotide	131-142 of Seq. Id. No. 1	6
	3 Amino Acids		7
4	33 Nucleotide	342-374 of Seq. Id. No. 1	8
	10 Amino Acids		9
5	15 Nucleotide	436-450 of Seq. Id. No. 1	10
	4 Amino Acids		11
6	42 Nucleotide	447-488 of Seq. Id. No. 1	12
	13 Amino Acids		13
7	42 Nucleotide	647-688 of Seq. Id. No. 1	14
	13 Amino Acids		15
8	63 Nucleotide	688-750 of Seq. Id. No. 1	16
	20 Amino Acids		17
9	45 Nucleotide	706-750 of Seq. Id. No. 1	18
	14 Amino Acids		19
10	33 Nucleotide	718-750 of Seq. Id. No. 1	20
	10 Amino Acids		21
11	24 Nucleotide	727-750 of Seq. Id. No. 1	22
	7 Amino Acids		23
12	106 Nucleotide	747-842 of Seq. Id. No. 1	24
	35 Amino Acids		25

TABLE 3

OPEN READING FRAMES FOR CNI-00712

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	54 Nucleotide	20-73 of Seq. Id. No. 26	27
	17 Amino Acid		28
2	57 Nucleotide	86-142 of Seq. Id. No. 26	29
	18 Amino Acid		30
3	12 Nucleotide	228-239 of Seq. Id. No. 26	31
	3 Amino Acids		32
4	93 Nucleotide	249-341 of Seq. Id. No. 26	33
	30 Amino Acids		34
5	30 Nucleotide	304-333 of Seq. Id. No. 26	35
	9 Amino Acids		36
6	309 Nucleotide	338-646 of Seq. Id. No. 26	37
	102 Amino Acids		38
7	93 Nucleotide	360-452 of Seq. Id. No. 26	39
	30 Amino Acids		40
8	261 Nucleotide	386-646 of Seq. Id. No. 26	41
	86 Amino Acids		42
9	57 Nucleotide	396-452 of Seq. Id. No. 26	43
	18 Amino Acids		44
10	195 Nucleotide	452-646 of Seq. Id. No. 26	45
	64 Amino Acids		46
11	480 Nucleotide	456-935 of Seq. Id. No. 26	47
	159 Amino Acids		48
12	141 Nucleotide	506-646 of Seq. Id. No. 26	49
	46 Amino Acids		50
13	420 Nucleotide	516-935 of Seq. Id. No. 26	51
	139 Amino Acids		52
14	399 Nucleotide	537-935 of Seq. Id. No. 26	53
	132 Amino Acids		54
15	81 Nucleotide	566-646 of Seq. Id. No. 26	55
	26 Amino Acids		56
16	348 Nucleotide	588-935 of Seq. Id. No. 26	57
	115 Amino Acids		58
17	27 Nucleotide	620-646 of Seq. Id. No. 26	59
	8 Amino Acids		60
18	303 Nucleotide	633-935 of Seq. Id. No. 26	61
	100 Amino Acids		62

19	84 Nucleotide	796-879 of Seq. Id. No. 26	63
	27 Amino Acids		64
20	36 Nucleotide	900-935 of Seq. Id. No. 26	65
	11 Amino Acids		66
21	24 Nucleotide	991-1014 of Seq. Id. No. 26	67
	7 Amino Acids		68
22	18 Nucleotide	997-1014 of Seq. Id. No. 26	69
	5 Amino Acids		70
23	33 Nucleotide	1007-1039 of Seq. Id. No. 26	71
	10 Amino Acids		72
24	15 Nucleotide	1041-1055 of Seq. Id. No. 26	73
	4 Amino Acids		74

TABLE 4

OPEN READING FRAMES FOR CNI-00714

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	1239 Nucleotide	29-1267 of Seq. Id. No. 75	76
	412 Amino Acid		77
2	105 Nucleotide	126-230 of Seq. Id. No. 75	78
	34 Amino Acid		79
3	1092 Nucleotide	76-1267 of Seq. Id. No. 75	80
	363 Amino Acids		81
4	18 Nucleotide	360-377 of Seq. Id. No. 75	82
	5 Amino Acids		83
5	69 Nucleotide	393-461 of Seq. Id. No. 75	84
	22 Amino Acids		85
6	24 Nucleotide	546-569 of Seq. Id. No. 75	86
	7 Amino Acids		87
7	96 Nucleotide	573-668 of Seq. Id. No. 75	88
	31 Amino Acids		89
8	87 Nucleotide	582-668 of Seq. Id. No. 75	90
	28 Amino Acids		91
9	600 Nucleotide	668-1267 of Seq. Id. No. 75	92
	199 Amino Acids		93
10	159 Nucleotide	684-842 of Seq. Id. No. 75	94
	52 Amino Acids		95
11	510 Nucleotide	758-1267 of Seq. Id. No. 75	96
	169 Amino Acids		97
12	51 Nucleotide	792-842 of Seq. Id. No. 75	98
	16 Amino Acids		99
13	336 Nucleotide	932-1267 of Seq. Id. No. 75	100
	111 Amino Acids		101
14	33 Nucleotide	1017-1049 of Seq. Id. No. 75	102
	10 Amino Acids		103
15	216 Nucleotide	1052-1267 of Seq. Id. No. 75	104
	71 Amino Acids		105
16	60 Nucleotide	1080-1139 of Seq. Id. No. 75	106
	19 Amino Acids		107
17	48 Nucleotide	1092-1139 of Seq. Id. No. 75	108
	15 Amino Acids		109
18	30 Nucleotide	1110-1139 of Seq. Id. No. 75	110
	9 Amino Acids		111

19	141 Nucleotide	1127-1267 of Seq. Id. No. 75	112
	46 Amino Acids		113
20	132 Nucleotide	1136-1267 of Seq. Id. No. 75	114
	43 Amino Acids		115
21	90 Nucleotide	1167-1256 of Seq. Id. No. 75	116
	29Amino Acids		117
22	72 Nucleotide	1185-1256 of Seq. Id. No. 75	118
	23 Amino Acids		119
23	57 Nucleotide	1211-1267 of Seq. Id. No. 75	120
	18 Amino Acids		121
24	15 Nucleotide	1253-1267 of Seq. Id. No. 75	122
	4 Amino Acids		123
25	45 Nucleotide	1283-1327 of Seq. Id. No. 75	124
	14 Amino Acids		125
26	132 Nucleotide	1411-1542 of Seq. Id. No. 75	126
	43 Amino Acids		127
27	105 Nucleotide	1438-1542 of Seq. Id. No. 75	128
	34 Amino Acids		129
28	75 Nucleotide	1493-1567 of Seq. Id. No. 75	130
	24 Amino Acids		131
29	39 Nucleotide	1573-1611 of Seq. Id. No. 75	132
	12 Amino Acids		133
30	33 Nucleotide	1528-1660 of Seq. Id. No. 75	134
	10 Amino Acids		135

TABLE 5

OPEN READING FRAMES FOR CNI-00715

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	39 Nucleotide	34-72 of Seq. Id. No. 136	137
	12 Amino Acid		138
2	24 Nucleotide	38-61 of Seq. Id. No. 136	139
	7 Amino Acid		140
3	138 Nucleotide	93-230 of Seq. Id. No. 136	141
	45 Amino Acids		142
4	93 Nucleotide	138-230 of Seq. Id. No. 136	143
	30 Amino Acids		144
5	72 Nucleotide	145-216 of Seq. Id. No. 136	145
	23 Amino Acids		146
6	57 Nucleotide	160-216 of Seq. Id. No. 136	147
	18 Amino Acids		148
7	30 Nucleotide	352-381 of Seq. Id. No. 136	149
	9 Amino Acids		150
8	75 Nucleotide	399-473 of Seq. Id. No. 136	151
	24 Amino Acids		152

TABLE 6

OPEN READING FRAMES FOR CNI-00716

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	108 Nucleotide	53-160 of Seq. Id. No. 153	154
	35 Amino Acid		155
2	99 Nucleotide	62-160 of Seq. Id. No. 153	156
	32 Amino Acid		157
3	21 Nucleotide	205-225 of Seq. Id. No. 153	158
	6 Amino Acids		159
4	75 Nucleotide	226-300 of Seq. Id. No. 153	160
	24 Amino Acids		161
5	48 Nucleotide	253-300 of Seq. Id. No. 153	162
	15 Amino Acids		163
6	42 Nucleotide	259-300 of Seq. Id. No. 153	164
	13 Amino Acids		165
7	99 Nucleotide	358-456 of Seq. Id. No. 153	166
	32 Amino Acids		167
8	63 Nucleotide	394-456 of Seq. Id. No. 153	168
	20 Amino Acids		169
9	39 Nucleotide	418-456 of Seq. Id. No. 153	170
	12 Amino Acids		171
10	177 Nucleotide	459-635 of Seq. Id. No. 153	172
	58 Amino Acids		173
11	27 Nucleotide	574-600 of Seq. Id. No. 153	174
	8 Amino Acids		175
12	75 Nucleotide	604-678 of Seq. Id. No. 153	176
	24 Amino Acids		177
13	33 Nucleotide	693-725 of Seq. Id. No. 153	178
	10 Amino Acids		179
14	30 Nucleotide	696-725 of Seq. Id. No. 153	180
	9 Amino Acids		181
15	42 Nucleotide	730-771 of Seq. Id. No. 153	182
	14 Amino Acids		183

TABLE 7

OPEN READING FRAMES FOR CNI-00717

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	819 Nucleotide	80-898 of Seq. Id. No. 184	185
	272 Amino Acid		186
2	774Nucleotide	125-898 of Seq. Id. No. 184	187
	257 Amino Acid		188
3	717 Nucleotide	182-898 of Seq. Id. No. 184	189
	238 Amino Acids		190
4	699 Nucleotide	200-898 of Seq. Id. No. 184	191
	232 Amino Acids		192
5	696 Nucleotide	203-898 of Seq. Id. No. 184	193
	231 Amino Acids		194
6	72 Nucleotide	279-350 of Seq. Id. No. 184	195
	23 Amino Acids		196
7	66 Nucleotide	285-350 of Seq. Id. No. 184	197
	21 Amino Acids		198
8	57 Nucleotide	294-350 of Seq. Id. No. 184	199
	18 Amino Acids		200
9	51 Nucleotide	369-419 of Seq. Id. No. 184	201
	16 Amino Acids		202
10	306 Nucleotide	423-728 of Seq. Id. No. 184	203
	101 Amino Acids		204
11	282 Nucleotide	447-728 of Seq. Id. No. 184	205
	93 Amino Acids		206
12	231 Nucleotide	498-728 of Seq. Id. No. 184	207
	76 Amino Acids		208
13	213 Nucleotide	516-728 of Seq. Id. No. 184	209
	70 Amino Acids		210
14	195 Nucleotide	534-728 of Seq. Id. No. 184	211
	64 Amino Acids		212
15	189 Nucleotide	540-728 of Seq. Id. No. 184	213
	62 Amino Acids		214
16	174 Nucleotide	555-728 of Seq. Id. No. 184	215
	57 Amino Acids		216
17	156 Nucleotide	573-728 of Seq. Id. No. 184	217
	51 Amino Acids		218
18	126 Nucleotide	603-728 of Seq. Id. No. 184	219
	41 Amino Acids		220

19	117 Nucleotide	612-728 of Seq. Id. No. 184	221
	38 Amino Acids		222
20	96 Nucleotide	633-728 of Seq. Id. No. 184	223
	31 Amino Acids		224
21	48 Nucleotide	681-728 of Seq. Id. No. 184	225
	15 Amino Acids		226
22	42 Nucleotide	687-728 of Seq. Id. No. 184	227
	13 Amino Acids		228
23	78 Nucleotide	741-818 of Seq. Id. No. 184	229
	25 Amino Acids		230
24	60 Nucleotide	759-818 of Seq. Id. No. 184	231
	19 Amino Acids		232
25	48 Nucleotide	771-818 of Seq. Id. No. 184	233
	15 Amino Acids		234
26	36 Nucleotide	783-818 of Seq. Id. No. 184	235
	11 Amino Acids		236
27	84 Nucleotide	846-929 of Seq. Id. No. 184	237
	27 Amino Acids		238
28	69 Nucleotide	861-929 of Seq. Id. No. 184	239
	22 Amino Acids		240
29	66 Nucleotide	864-929 of Seq. Id. No. 184	241
	21 Amino Acids		242
30	75 Nucleotide	931-1005 of Seq. Id. No. 184	243
	24 Amino Acids		244
31	75 Nucleotide	1062-1136 of Seq. Id. No. 184	245
	24 Amino Acids		246
32	18 Nucleotide	1119-1136 of Seq. Id. No. 184	247
	5 Amino Acids		248
33	15 Nucleotide	1162-1176 of Seq. Id. No. 184	249
	4 Amino Acids		250
34	81 Nucleotide	1304-1384 of Seq. Id. No. 184	251
	26 Amino Acids		252
35	24 Nucleotide	1361-1384 of Seq. Id. No. 184	253
	7 Amino Acids		254
36	27 Nucleotide	1396-1422 of Seq. Id. No. 184	255
	8 Amino Acids		256
37	90 Nucleotide	1478-1567 of Seq. Id. No. 184	257
	29 Amino Acids		258
38	24 Nucleotide	1554-1577 of Seq. Id. No. 184	259
	7 Amino Acids		260

TABLE 8

OPEN READING FRAMES FOR CNI-00720

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	24 Nucleotide	62-85 of Seq. Id. No. 261	262
	7 Amino Acid		263
2	228 Nucleotide	88-315 of Seq. Id. No. 261	264
	75 Amino Acid		265
3	195 Nucleotide	121-315 of Seq. Id. No. 261	266
	64 Amino Acids		267
4	69 Nucleotide	247-315 of Seq. Id. No. 261	268
	22 Amino Acids		269
5	87 Nucleotide	321-407 of Seq. Id. No. 261	270
	28 Amino Acids		271
6	270 Nucleotide	376-645 of Seq. Id. No. 261	272
	89 Amino Acids		273
7	21 Nucleotide	593-559 of Seq. Id. No. 261	274
	6 Amino Acids		275
8	42 Nucleotide	604-645 of Seq. Id. No. 261	276
	13 Amino Acids		277
9	18 Nucleotide	623-640 of Seq. Id. No. 261	278
	5 Amino Acids		279
10	99 Nucleotide	651-749 of Seq. Id. No. 261	280
	32 Amino Acids		281
11	33 Nucleotide	661-693 of Seq. Id. No. 261	282
	10 Amino Acids		283
12	54 Nucleotide	742-795 of Seq. Id. No. 261	284
	17 Amino Acids		285
13	15 Nucleotide	1020-1034 of Seq. Id. No. 261	286
	4 Amino Acids		287
14	48 Nucleotide	1034-1081 of Seq. Id. No. 261	288
	15 Amino Acids		289
15	12 Nucleotide	1126-1137 of Seq. Id. No. 261	290
	3 Amino Acids		291

TABLE 9

OPEN READING FRAMES FOR CNI-00721

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	207 Nucleotide	112-318 of Seq. Id. No. 292	293
	68 Amino Acids		294
2	147 Nucleotide	172-318 of Seq. Id. No. 292	295
	48 Amino Acids		296
3	24 Nucleotide	236-259 of Seq. Id. No. 292	297
	7 Amino Acids		298
4	18 Nucleotide	345-362 of Seq. Id. No. 292	299
	5 Amino Acids		300
5	51 Nucleotide	352-402 of Seq. Id. No. 292	301
	16 Amino Acids		302
6	132 Nucleotide	362-493 of Seq. Id. No. 292	303
	43 Amino Acids		304
7	33 Nucleotide	370-402 of Seq. Id. No. 292	305
	10 Amino Acids		306
8	21 Nucleotide	382-402 of Seq. Id. No. 292	307
	6 Amino Acids		308
9	12 Nucleotide	426-437 of Seq. Id. No. 292	309
	3 Amino Acids		310
10	201 Nucleotide	589-789 of Seq. Id. No. 292	311
	66 Amino Acids		312
11	93 Nucleotide	738-830 of Seq. Id. No. 292	313
	30 Amino Acids		314
12	21 Nucleotide	776-796 of Seq. Id. No. 292	315
	6 Amino Acids		316
13	42 Nucleotide	789-830 of Seq. Id. No. 292	317
	13 Amino Acids		318
14	27 Nucleotide	840-866 of Seq. Id. No. 292	319
	8 Amino Acids		320
15	324 Nucleotide	866-1189 of Seq. Id. No. 292	321
	107 Amino Acids		322
16	78 Nucleotide	870-947 of Seq. Id. No. 292	323
	25 Amino Acids		324
17	54 Nucleotide	894-947 of Seq. Id. No. 292	325
	17 Amino Acids		326
18	30 Nucleotide	918-947 of Seq. Id. No. 292	327
	9 Amino Acids		328

19	24 Nucleotide	976-999 of Seq. Id. No. 292	329
	7 Amino Acids		330
20	66 Nucleotide	1057-1122 of Seq. Id. No. 292	331
	21 Amino Acids		332
21	15 Nucleotide	1108-1122 of Seq. Id. No. 292	333
	4 Amino Acids		334
22	69 Nucleotide	1346-1414 of Seq. Id. No. 292	335
	22 Amino Acids		336
23	63 Nucleotide	1352-1414 of Seq. Id. No. 292	337
	20 Amino Acids		338
24	15 Nucleotide	1400-1414 of Seq. Id. No. 292	339
	4 Amino Acids		340
25	18 Nucleotide	1491-1508 of Seq. Id. No. 292	341
	5 Amino Acids		342
26	42 Nucleotide	1523-1564 of Seq. Id. No. 292	343
	13 Amino Acids		344
27	15 Nucleotide	1528-1542 of Seq. Id. No. 292	345
	4 Amino Acids		346
28	111 Nucleotide	1647-1757 of Seq. Id. No. 292	347
	36 Amino Acids		348
29	87 Nucleotide	1654-1740 of Seq. Id. No. 292	349
	28 Amino Acids		350
30	24 Nucleotide	1826-1849 of Seq. Id. No. 292	351
	7 Amino Acids		352
31	12 Nucleotide	1859-1870 of Seq. Id. No. 292	353
	3 Amino Acids		354
32	51 Nucleotide	1867-1917 of Seq. Id. No. 292	355
	16 Amino Acids		356
33	57 Nucleotide	1881-1937 of Seq. Id. No. 292	357
	18 Amino Acids		358

TABLE 10

OPEN READING FRAMES FOR CNI-00723

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	36 Nucleotide	217-252 of Seq. Id. No. 359	360
	11 Amino Acids		361
2	48 Nucleotide	288-335 of Seq. Id. No. 359	362
	15 Amino Acids		363
3	18 Nucleotide	332-349 of Seq. Id. No. 359	364
	5 Amino Acids		365
4	63 Nucleotide	393-455 of Seq. Id. No. 359	366
	20 Amino Acids		367
5	24 Nucleotide	412-435 of Seq. Id. No. 359	368
	7 Amino Acids		369
6	51 Nucleotide	439-489 of Seq. Id. No. 359	370
	16 Amino Acids		371
7	1473 Nucleotide	489-1961 of Seq. Id. No. 359	372
	490 Amino Acids		373
8	1467 Nucleotide	495-1961 of Seq. Id. No. 359	374
	488 Amino Acids		375
9	90 Nucleotide	544-633 of Seq. Id. No. 359	376
	29 Amino Acids		377
10	78 Nucleotide	556-633 of Seq. Id. No. 359	378
	25 Amino Acids		379
11	63 Nucleotide	571-633 of Seq. Id. No. 359	380
	20 Amino Acids		381
12	33 Nucleotide	614-646 of Seq. Id. No. 359	382
	10 Amino Acids		383
13	12 Nucleotide	622-633 of Seq. Id. No. 359	384
	3 Amino Acids		385
14	42 Nucleotide	634-675 of Seq. Id. No. 359	386
	13 Amino Acids		387
15	1260 Nucleotide	702-1961 of Seq. Id. No. 359	388
	419 Amino Acids		389
16	45 Nucleotide	736-780 of Seq. Id. No. 359	390
	14 Amino Acids		391
17	108 Nucleotide	740-847 of Seq. Id. No. 359	392
	35 Amino Acids		393
18	1128 Nucleotide	834-1961 of Seq. Id. No. 359	394
	375 Amino Acids		395

19	1017 Nucleotide	945-1961 of Seq. Id. No. 359	396
	338 Amino Acids		397
20	15 Nucleotide	986-1000 of Seq. Id. No. 359	398
	4 Amino Acids		399
21	30 Nucleotide	1000-1029 of Seq. Id. No. 359	400
	9 Amino Acids		401
22	936 Nucleotide	1026-1961 of Seq. Id. No. 359	402
	311 Amino Acids		403
23	12 Nucleotide	1061-1072 of Seq. Id. No. 359	404
	3 Amino Acids		405
24	39 Nucleotide	1069-1107 of Seq. Id. No. 359	406
	12 Amino Acids		407
25	33 Nucleotide	1075-1107 of Seq. Id. No. 359	408
	10 Amino Acids		409
26	870 Nucleotide	1092-1961 of Seq. Id. No. 359	410
	289 Amino Acids		411
27	54 Nucleotide	1258-1311 of Seq. Id. No. 359	412
	17 Amino Acids		413
28	678 Nucleotide	1284-1961 of Seq. Id. No. 359	414
	225 Amino Acids		415
29	21 Nucleotide	1342-1362 of Seq. Id. No. 359	416
	6 Amino Acids		417
30	24 Nucleotide	1379-1402 of Seq. Id. No. 359	418
	7 Amino Acids		419
31	15 Nucleotide	1429-1443 of Seq. Id. No. 359	420
	4 Amino Acids		421
32	486 Nucleotide	1476-1961 of Seq. Id. No. 359	422
	161 Amino Acids		423
33	54 Nucleotide	1489-1542 of Seq. Id. No. 359	424
	17 Amino Acids		425
34	15 Nucleotide	1528-1542 of Seq. Id. No. 359	426
	4 Amino Acids		427
35	57 Nucleotide	1543-1599 of Seq. Id. No. 359	428
	18 Amino Acids		429
36	24 Nucleotide	1576-1599 of Seq. Id. No. 359	430
	7 Amino Acids		431
37	240 Nucleotide	1722-1961 of Seq. Id. No. 359	432
	79 Amino Acids		433
38	90 Nucleotide	1872-1961 of Seq. Id. No. 359	434
	29 Amino Acids		435
39	54 Nucleotide	1915-1968 of Seq. Id. No.359	436
	17 Amino Acids		437
40	39 Nucleotide	1993-2031 of Seq. Id. No. 359	438
	12 Amino Acids		439
41	309 Nucleotide	2004-2312 of Seq. Id. No. 359	440
	102 Amino Acids		441

42	21 Nucleotide	2011-2031 of Seq. Id. No. 359	442
	6 Amino Acids		443
43	204 Nucleotide	2109-2312 of Seq. Id. No. 359	444
	67 Amino Acids		445
44	198 Nucleotide	2115-2312 of Seq. Id. No. 359	446
	65 Amino Acids		447
45	57 Nucleotide	2198-2254 of Seq. Id. No. 359	448
	18 Amino Acids		449
46	231 Nucleotide	2269-2499 of Seq. Id. No. 359	450
	76 Amino Acids		451
47	216 Nucleotide	2284-2499 of Seq. Id. No. 359	452
	71 Amino Acids		453
48	153 Nucleotide	2300-2454 of Seq. Id. No. 359	454
	50 Amino Acids		455
49	30 Nucleotide	2423-2452 of Seq. Id. No. 359	456
	9 Amino Acids		457
50	48 Nucleotide	2452-2499 of Seq. Id. No. 359	458
	15 Amino Acids		459
51	15 Nucleotide	2522-2536 of Seq. Id. No. 359	460
	4 Amino Acids		461

TABLE 11

OPEN READING FRAME FOR CNI-00724

OPEN READING FRAME NUMBER	LENGTH	LOCATION	SEQUENCE ID. NO.
1	243 Nucleotide	567-809 of Seq. Id. No. 462	463
	80 Amino Acids		464

In a specific embodiment, the nucleic acid molecules comprise nucleic acids that encode an open reading frame of at least 3 contiguous amino acid residues from a full-length protein. In alternate embodiments, the nucleic acid molecules comprise an open reading frame which encodes at least about 5, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or more contiguous amino acid residues of a protein.

The sequence obtained from clones containing partial coding sequences or non-coding sequences can be used to obtain the entire coding region by using the RACE method, for example (Chenchik, et al., 1995, CLONTECHniques (X) 1: 5-8; Barnes, 1994, Proc. Natl. Acad. Sci. USA 91: 2216-2220; and Cheng et al., Proc. Natl. Acad. Sci. USA 91: 5695-5699). Oligonucleotides can be designed based on the sequence obtained from the partial clone that can amplify a reverse transcribed mRNA encoding the entire coding sequence. Alternatively, probes can be used to screen cDNA libraries prepared from an appropriate cell or cell line in which the protective sequence is transcribed.

With respect to allelic variants of protective sequences associated with a condition, disorder, or disease involving cell death, any and all such nucleotide variations and resulting amino acid polymorphisms or variations which are the result of natural allelic variation of the protective sequence are intended to be within the scope of the present invention. Such allelic variants include, but are not limited to, ones that do not alter the functional activity of the protective sequence product.

With respect to the cloning of additional allelic variants of the isolated protective sequence and homologues and orthologs from other species (*e.g.*, guinea pig, cow, mouse), the isolated protective sequences disclosed herein may be labeled and used to screen a cDNA library constructed from mRNA obtained from appropriate cells or tissues (*e.g.*, brain) derived from the organism (*e.g.*, guinea pig, cow and mouse) of interest. The hybridization conditions used generally should be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived, and can routinely be determined based on, *e.g.*, relative relatedness of the target and reference organisms.

Alternatively, the labeled fragment may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. Appropriate stringency conditions are well known to those of skill in the art as discussed above, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions, *see*, for

example, Sambrook, *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, N.Y.; and Ausubel, *et al.*, 1989-1999, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y., both of which are incorporated herein by reference in their entirety.

Additionally, the cloning of homologs and orthologs of the isolated protective sequence from other species (e.g. mouse) could also occur using the knowledge of syntenic regions and/or genes. Syntenic genes are genes which are believed to be located on the same chromosome because they are lost along with a marker gene which is known to be located on that chromosome. There are well-established genetic maps of specific chromosome regions that show syntenic regions between chromosomes of humans and other species that can be utilized, by one skilled in the art, for this purpose.

Further, a protective sequence allelic variant may be isolated from, for example, human nucleic acid, by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within the protective sequence product of interest. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue known or suspected to express a wild type or mutant protective sequence allele. In one embodiment, the allelic variant is isolated from an individual who has a condition, disorder, or disease involving cell death. Such variants are described in the examples below.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a protective nucleic acid sequence. The PCR fragment may then be used to isolate a full-length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology also may be utilized to isolate full-length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source. A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction. The hybrid may be digested with RNAase H and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of

cloning strategies which may be used, see *e.g.*, Sambrook *et al.*, 1989, *supra*, or Ausubel *et al.*, *supra*.

In cases where the isolated protective sequence is the normal, or wild type gene, this gene may be used to isolate mutant alleles of the protective sequence. Such an isolation is preferable in processes and disorders that are known or suspected to have a genetic basis. Mutant alleles may be isolated from individuals either known or suspected to have a genotype which contributes to symptoms of conditions, disorders, or diseases involving cell death. Mutant alleles and mutant allele products may then be utilized in the therapeutic and diagnostic assay systems described below.

A cDNA of the mutant protective sequence may be isolated, for example, by using PCR, a technique well known to those of skill in the art. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying the mutant allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal protective sequence. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant protective sequence to that of the normal protective sequence, the mutation(s) responsible for the loss or alteration of function of the mutant gene product can be ascertained.

Alternatively, a genomic or cDNA library can be constructed and screened using DNA or RNA, respectively, from a tissue known to or suspected of expressing the protective sequence of interest in an individual suspected of or known to carry the mutant allele. The normal protective sequence or any suitable fragment thereof may then be labeled and used as a probed to identify the corresponding mutant allele in the library. The clone containing this protective sequence may then be purified through methods routinely practiced in the art, and subjected to sequence analysis as described above in this Section.

Additionally, an expression library can be constructed utilizing DNA isolated from or cDNA synthesized from a tissue known to or suspected of expressing the protective sequence of interest in an individual suspected of or known to carry the mutant allele. In this manner, protective sequence products made by the tissue containing the putative mutant alleles may be expressed and screened using standard antibody screening techniques in

conjunction with antibodies raised against the normal protective sequence product, as described, below, in Section 5.3 (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) In cases where the mutation results in an expressed protective sequence product with altered function (*e.g.*, as a result of a missense mutation), a polyclonal set of antibodies are likely to cross-react with the mutant protective sequence product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis as described in this Section, above.

The invention also includes nucleic acid molecules, preferably DNA molecules that are the complements of the nucleic acids of the preceding paragraphs.

In certain embodiments, the protective nucleic acid molecules of the invention are present as part of protective nucleic acid molecules comprising nucleic acid sequences which do not contain heterologous (*e.g.*, cloning vector or expression vector) sequences. In other embodiments, the protective nucleic acid molecules of the invention further comprise vector sequences, *e.g.*, cloning vectors or expression vectors.

5.2 Protein Products of the Protective Sequences

Protective sequence products or fragments thereof of the invention can be prepared for a variety of uses, including but not limited to, prophylactic or therapeutic modulators of protective sequence product function, for the generation of antibodies, diagnostic assays, or for the identification of other cellular or extracellular protective sequence products involved in the regulation of conditions, disorders, or diseases involving cell death.

The protective sequence products of the invention include, but are not limited to, human protective sequence products and non-human protective sequence products, *e.g.*, mammalian (such as bovine or guinea pig), protective sequence products.

Protective sequence products of the invention, sometimes referred to herein as a "protective sequence protein" or "protective sequence polypeptide," includes those gene products encoded by any of up to six translational reading frames of the protective sequence sequences depicted in Table 1, as well as gene products encoded by other human allelic variants and non-human variants of protective sequence products which can be identified by the methods herein described. Among such protective sequence product variants are protective sequence products comprising amino acid residues encoded by polymorphisms of

such protective sequence products.

In addition, protective sequence products of the invention may include proteins that represent functionally equivalent gene products. Functionally equivalent protective sequence products may include, for example, protective sequence products encoded by one of the nucleic acid molecules described in Section 5.1, above. In preferred embodiments, such functionally equivalent protective sequence products are naturally occurring gene products. Functionally equivalent protective sequence products also include gene products which retain at least one of the biological activities of the protective sequence products described above, and/or which are recognized by and bind to antibodies (polyclonal or monoclonal) directed against protective sequence products of the invention.

Equivalent protective sequence products may contain deletions, including internal deletions, additions, including additions yielding fusion proteins, or substitutions of amino acid residues within and/or adjacent to the amino acid sequence encoded by the protective sequence sequences described, above, in Section 5.1. Generally, deletions will be deletions of single amino acid residues, or deletions of no more than about 2, 3, 4, 5, 10 or 20 amino acid residues, either contiguous or non-contiguous. Generally, additions or substitutions, other than additions which yield fusion proteins, will be additions or substitutions of single amino acid residues, or additions or substitutions of no more than about 2, 3, 4, 5, 10 or 20 amino acid residues, either contiguous or non-contiguous. Preferably, these modifications result in a "silent" change, in that the change produces a protective sequence product with the same activity as the original protective sequence product. However, nucleic acid changes resulting in amino acid additions or substitutions may also be made for the purpose of modifying the protective sequence product in order to generally enhance their use as therapeutic agents or components for assays, such modifications to include, but not be limited to, stabilizing the product against degradation, enhancing pharmacokinetic properties, modifying site tropisms at the level of cells, tissues, organs, or organisms.

Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine; positively charged (basic) amino acids include arginine, lysine and histidine; and negatively

charged (acidic) amino acids include aspartic acid and glutamic acid. Additionally, non-natural amino acids, including, but not limited to, D-amino acids may be used.

Alternatively, where alteration of function is desired, addition(s), deletion(s) or non-conservative alterations can produce altered, including reduced-activity, protective sequence products. Such alterations can, for example, alter one or more of the biological functions of the protective sequence product. Further, such alterations can be selected so as to generate protective sequence products which include, but are not limited to, products which are better suited for expression, scale up, *etc.* in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges.

Protein fragments and/or peptides of the invention may comprise at least as many contiguous amino acid residues as necessary to represent an epitope fragment (that is to be recognized by an antibody directed to the protein). Examples of such protein fragments and/or peptides of the invention are shown by the open reading frames of the protective sequences shown in Figures 4-13, and described in Tables 2-11, respectively. In one nonlimiting embodiment of the invention, such protein fragments or peptides comprise at least about 3 contiguous amino acid residues from a full-length protein. In alternate embodiments, the protein fragments and peptides of the invention can comprise about 5, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or more contiguous amino acid residues of a protein.

Peptides and/or proteins corresponding to one or more domains of the protein as well as fusion proteins in which a protein, or a portion of a protein such as a truncated protein or peptide or a protein domain, is fused to an unrelated protein are also within the scope of this invention. Such proteins and peptides can be designed on the basis of the nucleic acids disclosed in Section 5.1, above. Fusion proteins include, but are not limited to, IgFc fusions which stabilize the protein or peptide and prolong half-life *in vivo*; or fusions to any amino acid sequence which allows the fusion protein to be anchored to the cell membrane; or fusions to an enzyme, fluorescent protein, luminescent protein or a epitope tagged protein or peptide which provides a marker function.

The protein sequences described above can include a domain, which comprises a protein transduction domain which targets the protective sequence product for delivery to various tissues and more particularly across the brain blood barrier, using, for

example, the protein transduction domain of human immunodeficiency virus TAT protein (Schwarze *et al.*, 1999, Science 285: 1569-72).

The protein sequences described above can include a domain, which comprises a signal sequence that targets the gene product for secretion. As used herein, a signal sequence includes a peptide of at least about 15 or 20 amino acid residues in length which occurs at the N-terminus of secretory and membrane-bound proteins and which contains at least about 70% hydrophobic amino acid residues such as alanine, leucine, isoleucine, phenylalanine, proline, tyrosine, tryptophan or valine. In a preferred embodiment, a signal sequence contains at least about 10 to 40 amino acid residues, preferably about 19-34 amino acid residues and has at least about 60-80%, more preferably 65-75% and more preferably at least about 70% hydrophobic residues. A signal sequence serves to direct a protein containing such a sequence to a lipid bilayer.

A signal sequence of a polypeptide of the invention can be used to facilitate secretion and isolation of the secreted protein or other proteins of interest. Signal sequences are typically characterized by a core of hydrophobic amino acids, which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. Thus, the invention pertains to the described polypeptides having a signal sequence (that is, "immature" polypeptides), as well as to the signal sequences themselves and to the polypeptides in the absence of a signal sequence (*i.e.*, the "mature" cleavage products). It is to be understood that polypeptides of the invention can further comprise polypeptides comprising any signal sequence having characteristics as described above and a mature polypeptide sequence.

In one embodiment, a nucleic acid sequence encoding a signal sequence of the invention can be operably linked in an expression vector to a protein of interest, such as a protein which is ordinarily not secreted or is otherwise difficult to isolate. The signal sequence directs secretion of the protein, such as from a eukaryotic host into which the expression vector is transformed, and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods. Alternatively, the signal sequence can be linked to the protein of interest using a sequence which facilitates purification, such as with a GST domain.

Finally, the proteins of the invention also include protein sequences wherein domains encoded by any transcriptional or post-transcriptional, and/or translational or post-

translational modifications, or fragments thereof, have been deleted. The polypeptides of the invention can further comprise posttranslational modifications, including, but not limited to glycosylations, acetylations and myrisalations.

The protective sequence products, peptide fragments thereof and fusion proteins thereof may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protective sequence products, polypeptides, peptides, fusion peptide and fusion polypeptides of the invention by expressing nucleic acid containing protective sequence sequences are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing protective sequence product coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. *See*, for example, the techniques described in Sambrook, *et al.*, 1989, *supra*, and Ausubel, *et al.*, 1989, *supra*. Alternatively, RNA capable of encoding protective sequence product sequences may be chemically synthesized using, for example, synthesizers. *See*, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, ed., IRL Press, Oxford.

A variety of host-expression vector systems may be utilized to express the protective sequence product coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the protective sequence product of the invention *in situ*. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing protective sequence product coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the protective sequence product coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the protective sequence product coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing protective sequence product coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived

from the genome of mammalian cells (*e.g.*, metallothionine promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the protective sequence product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of protective sequence product or for raising antibodies to protective sequence product, for example, vectors which direct the expression of high levels of fusion protein products which are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO J. 2:1791), in which the protective sequence product coding sequence may be ligated individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke and Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned protective sequence product can be released from the GST moiety.

In an insect system, *Autographa californica*, nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The protective sequence product coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of protective sequence product coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (*e.g.*, *see* Smith, *et al.*, 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the protective sequence product coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader

sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing protective sequence products in infected hosts. (*See, e.g.*, Logan and Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted protective sequence product coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire protective sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the protective sequence coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (*see* Bittner, *et al.*, 1987, *Methods in Enzymol.* 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3 and WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the protective sequence product may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators,

polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the protective sequence product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the protective sequence product.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, *Cell* 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, *et al.*, 1980, *Proc. Natl. Acad. Sci. USA* 77:3567; O'Hare, *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, *J. Mol. Biol.* 150:1); and hygro, which confers resistance to hygromycin (Santerre, *et al.*, 1984, *Gene* 30:147).

Alternatively, the expression characteristics of an endogenous protective sequence within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous protective sequence. For example, an endogenous protective sequence which is normally "transcriptionally silent", *i.e.*, a protective sequence which is normally not expressed, or is expressed only at very low levels in a cell line or microorganism, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed protective sequence product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous protective sequence may be activated by insertion of a promiscuous regulatory element which works across cell types.

Methods, which are well known to those skilled in the art, can be used to construct vectors containing the protective sequence operatively associated with appropriate

transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, and synthetic techniques. *See*, for example, the techniques described in Sambrook, *et al.*, 1992, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

The protective sequences may be associated operatively with a variety of different promoter/enhancer elements. The expression elements of these vectors may vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. The promoter may be in the form of the promoter that is associated naturally with the gene of interest. Alternatively, the DNA may be positioned under the control of a recombinant or heterologous promoter, *i.e.*, a promoter that is not associated normally with that gene. For example, tissue specific promoter/enhancer elements may be used to regulate the expression of the transferred DNA in specific cell types. Examples of transcriptional control regions which exhibit tissue specificity which have been described and could be used, include, but are not limited to: choline acetyltransferase (ChAT) gene control region which is active in cholinergic cells in the brain (Lonnerberg *et al.*, 1996, JBC 271:33358-65; Lonnerberg *et al.*, 1995, PNAS 92: 4046-50; Ibenez and Perrson, 1991 Eur. J. Neurosci. 3: 1309-15), mouse Thy-1.2 gene control region which is active in adult neurons including hippocampus, thalamus, cerebellum, cortex, RGC, DRG, and MN in the brain (Caroni, 1997, J Neurosci. Meth. 71: 3-9; Vidal *et al.*, 1990, EMBO J 9: 833-40), neuron specific enolase (NSE) gene control region which is active in pan-neuronal, neuron specific, deep layers of cerebral and neocortex (not in white matter) areas of the brain (Hannas-Djebbara *et al.*, 1997, Brain Res. Mol. Brain Res. 46: 91-9; Peel *et al.*, 1997, Gene Therapy 4: 16-24; Twyman *et al.*, 1997, J Mol Neurosci 8: 63-73; Forss-Petter *et al.*, 1990, Neuron 5:187-97), elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:42S-51S); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122); immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adams *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444); albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276); alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987,

Science 235:53-58); alpha-1-antitrypsin gene control region which is active in liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171); beta-globin gene control region which is active in myeloid cells (Magram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378). Promoters isolated from the genome of viruses which grow in mammalian cells (*e.g.*, CMV, RSV, vaccinia virus 7.5K, SV40, HSV, adenoviruses MLP, and MMTV LTR promoters) may be used, as well as promoters produced by recombinant DNA or synthetic techniques. Further, promoters specifically activated within bone, *i.e.*, the osteocalcin promoter, which is specifically activated within cells of osteoblastic lineage, may be used to target expression of nucleic acids within bone cells.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such that it is operatively linked with an endogenous protective sequence, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art, and described *e.g.*, in Chappel, U.S. Patent No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991.

Alternatively, utilizing an antibody specific for the fusion protein being expressed may readily purify any fusion protein. For example, a system described by Janknecht, *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+}-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The protective sequence products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys and chimpanzees may be used to generate transgenic animals. The term "transgenic," as used herein, refers to animals expressing protective sequences from a different species (*e.g.*, mice expressing human protective sequences), as well as animals which have been genetically

engineered to overexpress endogenous (*i.e.*, same species) sequences or animals which have been genetically engineered to no longer express endogenous protective sequences (*i.e.*, "knock-out" animals), and their progeny.

Any technique known in the art may be used to introduce a protective sequence transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe and Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten, *et al.*, 1985, *Proc. Natl. Acad. Sci., USA* 82:6148-6152); gene targeting in embryonic stem cells (Thompson, *et al.*, 1989, *Cell* 56:313-321); electroporation of embryos (Lo, 1983, *Mol. Cell. Biol.* 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, *Cell* 57:717-723) (For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115, 171-229).

Any technique known in the art may be used to produce transgenic animal clones containing a protective sequence transgene, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal or adult cells induced to quiescence (Campbell, *et al.*, 1996, *Nature* 380:64-66; Wilmut, *et al.*, *Nature* 385:810-813).

The present invention provides for transgenic animals which carry a protective sequence transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene also may be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (Lasko, *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:6232-6236). The regulatory sequences required for such a cell-type specific activation will depend on the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the cerebral transgene be integrated into the chromosomal site of the endogenous protective sequence, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleic acids homologous to the endogenous protective sequence are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleic acid of the endogenous protective sequence. The transgene also may be selectively introduced into a particular cell type, thus inactivating the endogenous protective sequence in only that cell type, by following, for example, the teaching of Gu, *et al.* (Gu, *et al.*, 1994, *Science* 265, 103-106). The regulatory sequences required for such a cell-type specific

inactivation will depend on the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant protective sequence may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis and RT-PCR (reverse transcriptase PCR). Samples of protective sequence-expressing tissue also may be evaluated immunocytochemically using antibodies specific for the transgene product.

Protective proteins can be used, *e.g.*, to treat cell death-related conditions, disorders, or diseases. Such protective sequence products include, but are not limited to, soluble derivatives such as peptides or polypeptides corresponding to one or more domains of the protective sequence product which are modified such that they are deleted for one or more hydrophobic domains. Alternatively, antibodies to the protein or anti-idiotypic antibodies which mimic the protective sequence product (including Fab fragments), modulators, antagonists or agonists can be used to treat cell death-related conditions, disorders, or diseases involving the protective sequence product. In yet another approach, nucleotide constructs encoding such protective sequence products can be used to genetically engineer host cells to express such protective sequence products *in vivo*; these genetically engineered cells can function as "bioreactors" in the body delivering a continuous supply of protective sequence product, peptides and soluble polypeptides.

5.3 Antibodies to the Protective Sequence Products

Described herein are methods for the production of antibodies capable of specifically recognizing one or more protective sequence product epitopes or epitopes of conserved variants or peptide fragments of the protective sequence products of the invention. Further, antibodies that specifically recognize mutant forms of the protective sequence products of the invention are encompassed by the invention. The terms "specifically bind" and "specifically recognize" refer to antibodies which bind to protective sequence product epitopes involved in conditions, disorders, or diseases involving cell death at a higher affinity

than they bind to protective sequence product epitopes not involved in such conditions, disorders, or diseases (*e.g.*, random epitopes).

Such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a protective sequence product in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal levels of protective sequence products, and/or for the presence of abnormal forms of such protective sequence products. Such antibodies also may be utilized in conjunction with, for example, compound screening schemes, as described, below, in Section 5.4.2, for the evaluation of the effect of test compounds on protective sequence product levels and/or activity. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described below, in Section 5.4.1.3., to evaluate, for example, the normal and/or engineered cells prior to their introduction into the patient.

Antibodies derived from the protective sequence or protective sequence product, including, but not limited to, antibodies and anti-idiotypic antibodies that mimic activity or function additionally may be used in methods for inhibiting abnormal protective sequence product activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods for protective sequence product-mediated conditions, disorders, or diseases.

For the production of antibodies against a protective sequence, various host animals may be immunized with a protective sequence or protective sequence product, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as protective sequence

product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized with protective sequence product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4:72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison, *et al.*, 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger, *et al.*, 1984, *Nature* 312:604-608; Takeda, *et al.*, 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. (See, *e.g.*, Cabilly *et al.*, U.S. Patent No. 4,816,567; and Boss *et al.*, U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.)

In addition, techniques have been developed for the production of humanized antibodies. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.) An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarily determining regions (CDRs). The extent of the framework region and CDRs have been precisely defined (see, "Sequences of Proteins of Immunological Interest", Kabat, E. *et al.*, U.S. Department of Health and Human Services (1983)). Briefly, humanized antibodies are antibody molecules from non-human species having one or more CDRs from the non-human species and a framework region from a human immunoglobulin molecule.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, *Science* 242:423-426; Huston, *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward, *et al.*, 1989, *Nature* 334:544-546) can be adapted to produce single chain antibodies against protective sequence products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the $F(ab')_2$ fragments, which can be produced by pepsin digestion of the antibody molecule and the Fab fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse, *et al.*, 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

5.4 Uses of the Protective Sequences, Protective Sequence Products and Antibodies

Described herein are various uses and applications of protective sequences, protective sequence products, including peptide fragments and fusion proteins thereof and of antibodies and anti-idiotypic antibodies derived from the protective sequence products and peptide fragments thereof. The application relates to compositions and methods for the treatment of conditions, disorders, or diseases involving cell death. Such applications include, but are not limited to, the prophylactic or therapeutic use of protective sequences which, when introduced into a cell predisposed to undergo cell death or in the process of dying, to prevent, delay, or rescue a cell, cells, tissue, organs, or organisms from dying, as described below in Section 5.4.1

Additionally, such applications include methods for the treatment of conditions, disorders, or diseases involving cell death, including, but not limited to, those associated with the central nervous system including neurological and psychiatric conditions, disorders, or diseases, and others as described below, in Section 5.4.1.1, and for the identification of compounds which modulate the expression of the protective sequence and/or the synthesis or activity of the protective sequence product, as described below, in Section 5.4.1. Such compounds can include, for example, other cellular products that are involved in

such processes as the regulation of cell death. These compounds can be used, for example, in the amelioration of conditions, disorders, or diseases involving cell death.

One example of the type of injury that can cause cell death in neuronal cells is stroke, which often is the result of ischemic injury. A relatively broad time window (8 hours to perhaps several days or longer) exists between the onset of ischemic injury (*i.e.* cessation or marked reduction in blood flow) before most neural cells actually die. There are many complex pathways and perhaps hundreds of different signaling molecules which are likely to be involved, leaving many different intervention points each with the potential to prevent, delay, arrest and reverse the cell death program. These delayed biochemical intervention points represent ideal clinical intervention points as they correspond to the time period during which most stroke patients present for medical treatment.

Many current medications for the treatment of stroke affect the physical and biochemical events that are acutely related to the initial onset of stroke, and, thus, must be administered soon after the biochemical cascades begin. These approaches all suffer from the necessity of administering the drugs within a very brief time window following a stroke. However, many stroke patients do not even realize that they have suffered from a stroke until a time point at which many of the current treatments are ineffective. This is because many stroke patients often do not present at the emergency room prior to the passing of at least 13 hours from the onset of the stroke. The methods and compounds of the present invention, however, can be administered during the broader time window between stroke and the onset of the pathways leading to cell death.

In addition to stroke, a variety of other conditions, disorders, and diseases lead to the activation of the same biochemical cascades which lead to neuronal cell death in stroke. There is growing evidence that numerous other disease states that induce cell death programs are related to those induced by stroke. Cell death programs have been increasingly implicated in Alzheimer's disease, a well-known neurodegenerative condition which leads to substantial loss of specific neuronal populations in the neocortex and hippocampus. Vascular dementia (multi-infarct dementia) is another disorder in which stroke-like cell death pathways are active. In vascular dementia, a repetitive process of small blood vessel diseases induces regional brain cell death, leading to a progressive loss of cognitive abilities. A partial list of other brain diseases which activate brain cell death pathways similar to those observed in stroke include, but are not limited to, Parkinson's disease, traumatic injury, Down's syndrome, Huntington's disease, HIV infection and intracranial infections.

One notable example from the preceding list is physical trauma to the nervous system. Although such trauma can be caused by a multitude of different physical insults to the head, neck, spine and other parts of the nervous system, all result in focal damage to, and death of, neural tissue and its component cells. Focally damaged areas behave similarly to stroke-induced infarcts in that a wider area of neural damage and death, a penumbra, is induced via biochemical and cellular mechanisms which are similar or identical to those occurring in stroke.

While, for clarity, the uses described in this section are primarily uses related to conditions, disorders, or diseases involving cell death, it is to be noted that each of the diagnostic and therapeutic treatments described herein can be additionally utilized in connection with other defects associated with the protective sequences of the invention.

Additionally, described herein are various applications of protective sequences, protective sequence products, genes, gene products, and/or their regulatory elements, including, but not limited to, prognostic and diagnostic evaluation of conditions, disorders, or diseases as described below in Section 5.4.1.1.

A variety of methods can be employed for the diagnostic and prognostic evaluation of conditions, disorders, or diseases involving cell death and for the identification of subjects having a predisposition to such conditions, disorders, or diseases.

Since protective sequences or protective sequence products need not normally be involved in all conditions, disorders, or diseases involving cell death, methods of the invention include, for example, modulating the expression of the protective sequence and/or the activity of the protective sequence product for the treatment of conditions, disorders, or diseases involving cell death which are normally mediated by some other gene.

For cell death related conditions, disorders, or diseases in which the protective sequences or protective sequence products are involved normally, such diagnostic and prognostic methods may, for example, utilize reagents such as the protective nucleic acids described in Section 5.1, and antibodies directed against protective sequence products, including peptide fragments thereof, as described, above, in Section 5.3.

Specifically, such reagents may be used, for example, for:

(1) the detection of the presence of protective sequence mutations, or the detection of either over- or under-expression of the protective sequence relative to wild-type levels of expression;

(2) the detection of over- or under-abundance of protective sequence products

relative to wild-type abundance of the protective sequence product; and

(3) the detection of an aberrant level of protective sequence product activity relative to wild-type protective sequence product activity levels.

Protective nucleic acids can, for example, be used to diagnose a condition, disorder, or disease involving cell death using, for example, the techniques for mutation/polymorphism detection described above in Section 5.1.

Mutations at a number of different genetic loci may lead to phenotypes related to conditions, disorders, or diseases involving cell death. Ideally, the treatment of patients suffering from such conditions, disorders, or diseases will be designed to target the particular genetic loci containing the mutation mediating the condition, disorder, or disease. Genetic polymorphisms have been linked to differences in drug effectiveness. Thus, identification of alterations in protective sequence, protein or gene flanking regions can be utilized in pharmacogenetic methods to optimize therapeutic drug treatments.

In one embodiment of the present invention, therefore, alterations, *i.e.*, polymorphisms, in the protective sequence or protein encoded by genes comprising such polymorphisms, are associated with a drug or drugs' efficacy, tolerance or toxicity, and may be used in pharmacogenomic methods to optimize therapeutic drug treatments, including therapeutic drug treatments for one of the conditions, disorders, or diseases described herein contained in Section 5.4.1.1, *e.g.*, central nervous system conditions, disorders, or diseases. Such polymorphisms can be used, for example, to refine the design of drugs by decreasing the incidence of adverse events in drug tolerance studies, *e.g.*, by identifying patient subpopulations of individuals who respond or do not respond to a particular drug therapy in efficacy studies, wherein the subpopulations have a polymorphism associated with drug responsiveness or unresponsiveness. The pharmacogenomic methods of the present invention also can provide tools to identify new drug targets for designing drugs and to optimize the use of already existing drugs, *e.g.*, to increase the response rate to a drug and/or to identify and exclude non-responders from certain drug treatments (*e.g.*, individuals having a particular polymorphism associated with unresponsiveness or inferior responsiveness to the drug treatment) or to decrease the undesirable side effects of certain drug treatments and/or to identify and exclude individuals with marked susceptibility to such side effects (*e.g.*, individuals having a particular polymorphism associated with an undesirable side effect to the drug treatment).

In an embodiment of the present invention, polymorphisms in the protective sequence or flanking this sequence, or variations in protective sequence expression, or activity, *e.g.*, variations due to altered methylation, differential splicing or post-translational modification of the protective sequence product, may be utilized to identify an individual having a disease or condition resulting from a disorder involving cell death and thus define the most effective and safest drug treatment. Assays such as those described herein may be used to identify such polymorphisms or variations in protective sequence expression or activity. Once a polymorphism in the protective sequence or in a flanking sequence in linkage disequilibrium with a disorder-causing allelle, or a variation in protective sequence expression has been identified in an individual, an appropriate drug treatment can be prescribed to the individual.

For the detection of protective sequence mutations or polymorphisms, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of protective sequence expression or protective sequence products, any cell type or tissue in which the protective sequence is expressed may be utilized.

Nucleic acid-based detection techniques are described, below, in Section 5.4.1.4. Peptide detection techniques are described, below, in Section 5.4.1.5.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits. The invention therefore also encompasses kits for detecting the presence of a polypeptide or nucleic acid of the invention in a biological sample (*i.e.*, a test sample). Such kits can be used, *e.g.*, to determine if a subject is suffering from or is at increased risk of developing a condition, disorder, or disease associated with a disorder-causing allele, or aberrant expression or activity of a polypeptide of the invention. For example, the kit can comprise a labeled compound or agent capable of detecting the polypeptide or mRNA or DNA or protective sequence sequences, *e.g.*, encoding the polypeptide in a biological sample. The kit can comprise further a means for determining the amount of the polypeptide or mRNA in the sample (*e.g.*, an antibody that binds the polypeptide or an oligonucleotide probe that binds to DNA or mRNA encoding the polypeptide). Kits can also include instructions for observing that the tested subject is suffering from, or is at risk of developing, a condition, disorder, or disease associated with aberrant expression of the polypeptide if the amount of the polypeptide or mRNA encoding the polypeptide is above or below a normal level, or if the DNA correlates with presence of an allele which causes a condition, disorder, or disease.

For antibody-based kits, the kit can comprise, for example: (1) a first antibody (*e.g.*, attached to a solid support) which binds to a polypeptide of the invention; and, optionally, (2) a second, different antibody which binds to either the polypeptide or to the first antibody and is conjugated to a detectable agent.

For oligonucleotide-based kits, the kit can comprise, for example: (1) an oligonucleotide (*e.g.*, a detectably labeled oligonucleotide) which hybridizes to a nucleic acid sequence encoding a polypeptide of the invention, or (2) a pair of primers useful for amplifying a nucleic acid molecule encoding a polypeptide of the invention.

The kit also can comprise, for example, one or more buffering agents, preservatives or protein stabilizing agents. The kit also can comprise components necessary for detecting the detectable agent (*e.g.*, an enzyme or a substrate). The kit can contain also a control sample or a series of control samples that can be assayed and compared to the test sample. Each component of the kit usually is enclosed within an individual container and all of the various containers are within a single package along with instructions for observing whether the tested subject is suffering from or is at risk of developing a condition, disorder, or disease associated with polymorphisms which correlate with alleles which cause conditions, disorders, or diseases involving cell death, and/or aberrant levels of mRNA, polypeptides or activity.

Additionally, the application relates to the compositions and methods for the development of screening assays for the identification of compounds, described in Section 5.4.2 below, which interact with or modulate protective sequences, protective sequence products, genes, gene products, and/or their regulatory elements.

5.4.1 Composition and Methods for the Treatment of Conditions, Disorders, or Diseases Involving Cell Death

This application relates to compositions and methods for the treatment of conditions, disorders, or diseases involving cell death. Such applications include, but are not limited to, the prophylactic or therapeutic use of protective sequences, protective sequence products, genes, gene products, or the regulatory elements, target sequences, or variants of any of the aforementioned sequences or products, which, when introduced into a cell predisposed to undergo cell death or in the process of dying, prevent, delay, or rescue a cell, cells, tissue, organs, or organisms from dying. The application further relates to the methods and compositions whereby a condition, disorder, or disease involving cell death, including

but not limited to, the conditions, disorders, or diseases mentioned in Section 5.4.1.1, may be treated wherein such methods can comprise administering antibodies, antisense molecules or sequences, ribozyme molecules, or other inhibitors or modulators directed against such protective sequences, protective sequence products, genes, gene products, or the regulatory elements, target sequences, or variants of any of the aforementioned sequences or products.

The application relates to compositions and methods for those instances whereby the condition, disorder, or disease involving cell death results from protective sequence mutations, such methods can comprise supplying the subject with a nucleic acid molecule encoding an unimpaired protective sequence product such that an unimpaired protective sequence product is expressed and the cell, cells, tissue, organ, organism displaying symptoms of the condition, disorder, or disease is prevented, delayed, or rescued from death.

In another embodiment of methods for the treatment of conditions, disorders, or diseases involving cell death resulting from protective sequence mutations, such methods can comprise supplying the subject with a cell comprising a nucleic acid molecule which encodes an unimpaired protective sequence product such that the cell expresses the unimpaired protective sequence product and the cell, cells, tissue, organ, or organism displaying symptoms of the condition, disorder, or disease is prevented, delayed, or rescued from death.

In cases in which a loss of normal protective sequence product function results in the development of a condition, disorder, or disease involving cell death, an increase in protective sequence product activity would facilitate progress towards an asymptomatic state in individuals exhibiting a deficient level of protective sequence expression and/or gene product activity. Methods for enhancing the expression or synthesis of protective sequence product can include, for example, methods such as those described below, in Section 5.4.1.3.

Alternatively, symptoms of a condition, disorder, or disease involving cell death may be prevented, delayed, or rescued by administering a compound which decreases the level of protective sequence expression and/or gene product activity. Methods for inhibiting or reducing the level of protective sequence product synthesis or expression can include, for example, methods such as those described in Section 5.4.1.2.

In cases where the development of a condition, disorder, or disease involving cell death is due to a sequence or gene other than a protective sequence, modulating, including but not limited to, mimicking, agonizing, or antagonizing the expression of a

protective sequence and/or the activity of a protective sequence product, or their regulatory elements, can be used for the treatment of the condition, disorder, or disease involving cell death. This is because protective sequences are nucleic acid molecules comprising nucleic acid sequences which, when introduced into a cell predisposed to undergo cell death, prevent, delay, or rescue such cell death relative to a corresponding cell into which no exogenous protective sequence has been introduced.

The proteins and peptides which may be used in the methods of the invention include synthetic (*e.g.*, recombinant or chemically synthesized) proteins and peptides, as well as naturally occurring proteins and peptides. The proteins and peptides may have both naturally occurring and non-naturally occurring amino acid residues (*e.g.*, D-amino acid residues) and/or one or more non-peptide bonds (*e.g.*, imino, ester, hydrazide, semicarbazide, and azo bonds). The proteins or peptides may also contain additional chemical groups (*i.e.*, functional groups) present at the amino and/or carboxy termini, such that, for example, the stability, bioavailability, and/or inhibitory activity of the peptide is enhanced. Exemplary functional groups include hydrophobic groups (*e.g.* carbobenzoxyl, dansyl, and t-butyloxycarbonyl, groups), an acetyl group, a 9-fluorenylmethoxy-carbonyl group and macromolecular carrier groups (*e.g.*, lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates) including peptide groups. Additional proteins and peptides which may be used in the methods of the invention include those described in WO 99/59615, which is herein incorporated by reference in its entirety.

5.4.1.1 Examples of Conditions, Disorders, or Diseases Involving Cell Death

The types of conditions, disorders, or diseases which can be prevented, delayed, or rescued by the compounds and methods of the present invention include, but are not limited to, those associated with the central nervous system including neurological and psychiatric conditions, disorders, or diseases; those of the peripheral nervous system; conditions, disorders, or diseases caused by physical injury; conditions, disorders, or diseases of the blood vessels or heart; conditions, disorders, or diseases of the respiratory system; neoplastic conditions, disorders, or diseases; conditions, disorders, or diseases of blood cells; conditions, disorders, or diseases of the gastrointestinal tract; conditions, disorders, or diseases of the liver; conditions, disorders, or diseases of the pancreas; conditions, disorders, or diseases of the kidney; conditions, disorders, or diseases of the ureters, urethra or bladder; conditions, disorders, or diseases of the male genital system; conditions, disorders, or

diseases of the female genital tract; conditions, disorders, or diseases of the breast; conditions, disorders, or diseases of the endocrine system; conditions, disorders, or diseases of the thymus or pineal gland; conditions, disorders, or diseases of the skin or mucosa; conditions, disorders, or diseases of the musculoskeletal system; conditions, disorders, or diseases causing a fluid or hemodynamic derangement; inherited conditions, disorders, or diseases; conditions, disorders, or diseases of the immune system or spleen; conditions, disorders, or diseases caused by a nutritional disease; and conditions, disorders, or diseases typically occurring in infancy or childhood.

Conditions, disorders, or diseases involving the central nervous system include, but are not limited to, common pathophysiologic complications such as increased intracraneal pressure and cerebral herniation, septic embolism, cerebral edema, suppurative endovasculitis and hydrocephalus; infections such as meningitis, acute meningitis, acute lymphocytic meningitis, chronic meningitis, purulent meningitis, syphilitic gumma, encephalitis, cerebral abscess, epidural abscess, subdural abscess, brain abscess, viral encephalitis, acute viral encephalitis, encephalomeningitis, aseptic meningitis, post-infectious encephalitis, subacute encephalitis, chronic encephalitis, chronic meningitis, chronic encephalomeningitis, slow virus diseases and unconventional agent encephalopathies; protozoal infections such as malaria, toxoplasmosis, amebiasis and trypanosomiasis; rickettsial infections such as typhus and Rocky Mountain spotted fever; metazoal infections such as echinococcosis and cysticercosis; vascular diseases such as ischemic encephalopathy, cerebral infarction, intracranial hemorrhage, intraparenchymal hemorrhage, subarachnoid hemorrhage, mixed intraparenchymal and subarachnoid hemorrhage; conditions involving the eye such as macular degeneration, glaucoma, retinopathy of prematurity, retinitis pigmentosa, diabetic retinopathy, or other traumatic injuries to the retina or optic nerve; trauma such as epidural hematoma, subdural hematoma, parenchymal injuries; tumors such as primary intrachranial tumors, astrocytoma, oligodendroglioma, ependymoma, medulloblastoma and meningioma; degenerative diseases such as Altzheimer's disease, Huntington's disease, Parkinsonism, idiopathic Parkinson's disease and motor neuron disease; demyelinating diseases such as multiple sclerosis; nutritional, environmental and metabolic conditions, disorders, or diseases.

Conditions, disorders, or diseases of the peripheral nervous system include, but are not limited to, peripheral neuropathy, acute idiopathic polyneuropathy, diabetic neuropathy and peripheral nerve tumors.

Conditions, disorders, or diseases caused by physical injury include, but are not limited to, the direct, indirect, immediate, or delayed effects of: changes in temperature such as frostbite and thermal burns; an increase in atmospheric pressure such as air blast or immersion blast caused by an explosion; a decrease in atmospheric pressure such as caisson disease or high-altitude hypoxia; mechanical violence from penetrating or non-penetrating traumatic injury; electromechanical energy such as radiation injury from either charged particles or electromagnetic waves; electrocution or non-ionizing radiation such as radio waves, microwaves, laser light or ultrasound.

Conditions, disorders, or diseases of the blood vessels or heart include, but are not limited to, hypertension (high blood pressure), heart failure; ischemic or atherosclerotic heart disease; myocardial infarction; cardiac arrest; hypertensive heart disease; cor pulmonale; valvular heart disease such as that caused by rheumatic fever, aortic valve stenosis, mitral annulus calcification, carcinoid heart disease, nonbacterial thrombotic endocarditis, or nonbacterial verrucous endocarditis; infectious endocarditis caused by organisms including, but not limited to, Streptococcus species, Staphylococcus species, enterococci, pneumococci, gram-negative rods, Candida species, Aspergillus species, or culture-negative endocarditis; congenital heart disease such as atrial septal defect, ventricular septal defect, patent ductus arteriosis, coarctation of the aorta, Tetralogy of Fallot, tricuspid atresia, pulmonary stenosis or atresia, aortic stenosis or atresia, bicuspid aortic valve, or hypoplastic left heart syndrome; cardiomyopathy; pericarditis; pericardial effusion; rheumatoid heart disease; congenital anomalies of the blood vessels; arteriosclerosis including, but not limited to atherosclerosis, Monckeberg's medial calcific stenosis, hyaline arteriosclerosis, or hyperplastic arteriosclerosis; one or more of the vasculidities including, but not limited to, polyarteritis nodosa, hypersensitivity angiitis, Wegener's granulomatosis, giant cell (temporal) arteritis, Takayasu's arteritis, Kawasaki's disease, thromboangiitis obliterans, infectious vasculitis, Raynaud's disease; arteriosclerotic aortic aneurysm; syphilitic aortic aneurysm; dissecting aortic aneurysm; varicose veins; thrombophlebitis; lymphangitis; lymphedema; telangiectases; or arteriovenous malformations (AVM).

Conditions, disorders, or diseases of the respiratory system include, but are not limited to, pulmonary congestion; heart failure; embolism; infarction; pulmonary hypertension; adult respiratory distress syndrome (ARDS); obstructive lung disease; restrictive lung disease; chronic obstructive pulmonary disease; asthma; sarcoidosis; diffuse interstitial or infiltrative lung diseases including, but not limited to, idiopathic pulmonary

fibrosis, pneumoconiosis, hypersensitivity pneumonitis, Goodpasture's syndrome, idiopathic pulmonary hemosiderosis, collagen-vascular diseases, or pulmonary eosinophilia; serofibrinous pleuritis; suppurative pleuritis; hemorrhagic pleuritis; pleural effusions; pneumothorax; hemothorax or pneumohemothorax.

Neoplastic conditions, disorders, or diseases include, but are not limited to, benign tumors composed of one parenchymal cell type such as fibromas, myxomas, lipomas, hemangiomas, meningiomas, leiomyomas, adenomas, nevi, moles, or papillomas; benign mixed tumors derived from one germ layer such as a mixed tumor of salivary gland origin; benign mixed tumors derived from more than one germ layer such as a teratoma; primary malignant tumors or metastases of malignant tumors composed of one parenchymal cell type such as sarcomas, Ewing's tumor, leukemia, myeloma, histiocytosis X, Hodgkin's disease, lymphomas, carcinomas, melanomas, bronchial adenoma, small cell lung cancer, or seminoma; primary malignant tumors or metastases of mixed malignant tumors derived from one germ layer such as Wilms' tumor or malignant mixed salivary gland tumor; primary malignant tumor or metastases of mixed malignant tumors derived from one germ layer such as malignant teratoma or teratocarcinoma; undifferentiated benign tumor or undifferentiated malignant tumor.

Conditions, disorders, or diseases of blood cells include, but are not limited to, anemia due to one or more of the following conditions: acute blood loss, chronic blood loss, hemolytic anemia, sickle cell disease, thalassemia syndromes, autoimmune hemolytic anemia, traumatic anemia, or diminished erythropoesis from megaloblastic anemia, iron deficiency, aplastic anemia, idiopathic bone marrow failure; polycythemia; hemorrhagic diatheses related to increased vascular fragility; hemorrhagic diatheses related to a reduction in platelets; idiopathic or thrombotic thrombocytopenic purpura; hemorrhagic diatheses related to defective platelet function; hemorrhagic diatheses related to abnormalities in clotting factor(s); disseminated intravascular coagulation (DIC); neutropenia; agranulocytosis; leukocytosis; plasma cell dyscrasias such as myeloma, Waldenstrom's macroglobulinemia, or heavy-chain disease; or histiocytosis.

Conditions, disorders, or diseases of the gastrointestinal tract include, but are not limited to, congenital anomalies such as atresia, fistulas, or stenosis; periodontal disease; periapical disease; xerostomia; necrotizing sialometaplasia; esophageal rings or webs; hernia; Mallory-Weiss syndrome; esophagitis; diverticulosis; diverticulitis; scleroderma; esophageal varices; acute or chronic gastritis; peptic ulcer; gastric erosion or ulceration; ischemic bowel

disease; infarction; embolism; Crohn's disease; obstruction from foreign bodies, hernia, adhesion, intussusception, or volvulus; ileus; megacolon; angoidysplasia; ulcerative colitis; psuedomembranous colitis; or polyps.

Conditions, disorders, or diseases of the liver include, but are not limited to, acute hepatic failure due to one of more of metabolic, circulatory, toxic, microbial, or neoplastic causes; chronic hepatic failure due to one or more of metabolic, circulatory, toxic, microbial, or neoplastic causes; hereditary hyperbilirubinemias; infarct; embolism; hepatic circulation thrombosis or obstruction; fulminant hepatic necrosis; portal hypertension; alcoholic liver disease; post-necrotic cirrhosis; biliary cirrhosis; cirrhosis associated with alpha-1-antitrypsin deficiency; Wilson's disease; or Reye's syndrome.

Conditions, disorders, or diseases of the pancreas include, but are not limited to, congenital aberrant pancreas, congenital anomalies of pancreatic ducts, stromal fatty infiltration, pancreatic atrophy, acute hemorrhagic pancreatitis, chronic pancreatitis, chronic calcifying pancreatitis, chronic obstructive pancreatitis, pancreatic psuedocyst, diabetes mellitus, or gestational diabetes.

Conditions, disorders, or diseases of the kidney include, but are not limited to, congenital anomalies; polycystic renal disease; dialysis-associated cystic disease; glomerular disease, including, but not limited to, acute glomerulonephritis, acute proliferative glomerulonephritis, rapidly progressive glomerulonephritis, postinfectious rapidly progressive glomerulonephritis, Goodpasture's syndrome, idiopathic rapidly progressive glomerulonephritis, nephrotic syndrome, membranous glomerulonephritis, lipoid nephrosis, focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, focal proliferative glomerulonephritis, chronic glomerulonephritis, or hereditary nephritis; acute tubular necrosis; acute renal failure; tubulointerstitial diseases including, but not limited to, pyelonephritis, drug-induced interstitial nephritis, analgesic nephritis, urate nephropathy, hypercalcemia and nephrocalcinosis, hypokalemic nephropathy, myeloma-induced tubulointerstitial disease, radiation nephritis, immunologically medicated tubulointerstitial disease; hypertension; malignant hypertension; renal artery stenosis; renal diseases secondary to microangiopathic hemolytic anemia; atheroembolic renal disease; sickle cell disease nephropathy; diffuse cortical necrosis; renal infarcts; obstructive uropathy; or urolithiasis.

Conditions, disorders, or diseases of the ureters, urethra or bladder include, but are not limited to, congenital anomalies; inflammatory diseases; physical obstruction by causes including, but not limited to calculi, strictures, neoplasia, blood clot, or pregnancy;

sclerosing retroperitonitis; acute cystitis; chronic cystitis; interstitial cystitis; emphysematous cystitis; eosinophilic cystitis; encrusted cystitis; fistula; or neurogenic bladder.

Conditions, disorders, or diseases of the male genital system include, but are not limited to, congenital anomalies; balanoposthitis; condyloma; phimosis; paraphimosis; dysplastic epithelial lesions; nonspecific epididymitis or orchitis; granulomatous orchitis; torsion of the testis or its vascular supply; granulomatous prostatitis; acute or chronic prostatitis; or benign prostatic hyperplasia.

Conditions, disorders, or diseases of the female genital tract include, but are not limited to, congenital anomalies, lichen scleroses, acute cervicitis, chronic cervicitis, cervical polyps; acute endometritis; chronic endometritis; endometriosis; dysfunctional uterine bleeding; endometrial hyperplasia; senile cystic endometrial atrophy; salpingitis; polycystic ovary disease; pre-eclampsia or eclampsia (toxemia of pregnancy); placentitis; threatened abortion; or ectopic pregnancy.

Conditions, disorders, or diseases of the breast include, but are not limited to, congenital anomalies, acute mastitis, chronic mastitis, galactocele, granulomas, traumatic fat necrosis, mammary duct ectasia, fibrocystic disease, sclerosing adenitis, epithelial hyperplasia, hypertrophy, or gynecomastia.

Conditions, disorders, or diseases of the endocrine system include, but are not limited to, congenital anomalies; Sheehan's pituitary necrosis; empty sella syndrome; hyperthyroidism (thyrotoxicosis) from causes including, but not limited to, Graves' disease, toxic multinodular goiter, toxic adenoma, acute or subacute thyroiditis, TSH-secreting tumor, neonatal thyrotoxicosis, iatrogenic thyrotoxicosis; Hashimoto's thyroiditis; hypothyroidism (cretinism or myxedema) from causes including, but not limited to, surgical or radioactive ablation, primary idiopathic myxedema, iodine deficiency, goitrogenic agents, hypopituitarism, hypothalamic lesions, TSH resistance, subacute thyroiditis, or chronic thyroiditis; diffuse nontoxic simple or multinodular goiter; multiple endocrine neoplasia syndromes; primary or secondary hyperparathyroidism; chief cell hyperplasia; clear cell hyperplasia; hypoparathyroidism; pseudo- and pseudopseudohypoparathyrodism; Addison's disease; Waterhouse-Friderichsen syndrome; secondary adrenocortical insufficiency; Cushing's syndrome; Conn's syndrome; or congenital adrenal hyperplasia.

Conditions, disorders, or diseases of the skin or mucosa include, but are not limited to, melanocytic proliferative disorders; inflammatory dermatoses including, but not limited to, eczematous dermatitis, urticaria, erythema multiforme, cutaneous necrotizing

vasculitis, cutaneous lupus erythematosus, graft-versus-host disease, panniculitis, acne vulgaris, rosacea, lichen planus, lichen sclerosus et atrophicus, pityriasis, psoriasis, or parapsoriasis; blistering diseases including, but not limited to, pemphigus, bullous pemphigoid, dermatitis herpetiformis, or porphyria.

Conditions, disorders, or diseases of the musculoskeletal system include, but are not limited to, muscular atrophy; segmental necrosis; myositis; muscular dystrophy, including, but not limited to, Duchenne type, Becker type, Fascioscapulohumeral, Limb-Girdle, myotonic dystrophy, or ocular myopathy; congenital myopathies; myasthenia gravis; traumatic myositis ossificans; nodular fasciitis; desmoid tumors; palmar fibromatosis; congenital bone disorders including, but not limited to, osteogenesis imperfecta, achondroplasia, osteopetrosis, osteochondromatosis, endochondromatosis; osteomyelitis; fractures; osteoporosis; osteomalacia; bony changes secondary to hyperparathyroidism; Paget's disease; hypertrophic osteoarthropathy; fibrous dysplasia; or nonossifying fibroma.

Conditions, disorders, or diseases causing a fluid or hemodynamic derangement include, but are not limited to, systemic edema; anasarca; edema from increased hydrostatic pressure including, but not limited to congestive heart failure, cirrhosis of the liver, constrictive pericarditis, venous obstruction; edema from reduced oncotic pressure including, but not limited to, cirrhosis of the liver, malnutrition, protein-losing renal disease, protein-losing gastroenteropathy, protein loss through increased vascular permeability; edema from lymphatic obstruction including, but not limited to, cancer, inflammatory injury, surgical injury, traumatic injury, or radiation injury; edema from increased osmotic tension in the interstitial fluid including, but not limited to, sodium retention from excessive salt intake or increased renal sodium retention, reduced renal perfusion, acute or chronic renal failure, acute or chronic renal insufficiency; edema from increased endothelial permeability including, but not limited to, inflammation, shock, burns, trauma, allergic reaction, immunologic reaction, or adult respiratory distress syndrome; ascites; pericardial effusion; hydrothorax; hyperemia; hemorrhage; mural thrombus or occlusive thrombus diminishing or obstructing vascular flow; phlebothrombosis; blood clot; embolism; thromboembolism; disseminated intravascular coagulation (DIC); amniotic fluid infusion; amniotic fluid embolism; systemic embolism disease; septic embolism; fat embolism; pulmonary embolism; air gas embolism (caisson disease or decompression sickness); anemic (white) infarction; hemorrhagic (red) infarction; cerebral infarction; septic infarction; ischemia; cardiogenic shock from conditions including, but not limited to, myocardial infarction, cardiac arrest,

cardiac rupture, cardiac tamponade, pulmonary embolism, cardiac valvular obstruction, or cardiac arrhythmias; hypovolemic shock from conditions including, but not limited to, hemorrhage, vomiting, diarrhea, diaphoresis, extensive injury to bone or soft tissues, burns, or accumulation of intraperitoneal fluid; shock due to peripheral blood pooling from conditions including, but not limited to, spinal cord injury, general anesthesia, regional anesthesia, local anesthesia, drug-induced ganglionic or adrenergic blockade, gram-negative septicemia, or gram-positive septicemia; anaphylaxis, or disseminated intravascular coagulation (DIC).

Inherited conditions, disorders, or diseases include, but are not limited to, Down's syndrome, Edwards' syndrome, Patau's syndrome, other trisomies, Cri du Chat syndrome, Klinefelter's syndrome, XYY syndrome, Turner's syndrome, Multi-X female syndrome, hermaphrodism or pseudohermaphrodism, Marfan's syndrome, neurofibromatosis, vonHippel-Lindau disease, familial hypercholesterolemia, albinism, alkaptonuria, Fabry's disease, Fragile-X syndrome, Ehlers-Danlos syndromes, inherited neoplastic syndromes, inherited autosomal dominant conditions, Huntington's disease, Alport's disease, sickle-cell disease, thalessemia, tuberous sclerosis, vonWillebrand's disease, polycystic kidney disease, Pompe's disease, GM1-gangliosidosis; Tay-Sachs disease, Sandhoff-Jatzkewitz disease, metachromatic leukodystrophy, multiple sufatase deficiency, Krabbe's disease, Gaucher's disease, Niemann-Pick disease, all types of mucopolysaccharidoses, I-cell disease, Hurler's polydystrophy, fucosidosis, mannosidosis, aspartylglycosaminuria, Wolman's disease, or acid phosphatase deficiency, inherited autosomal recessive conditions, inherited sex-linked conditions.

Conditions, disorders, or diseases of the immune system or spleen include, but are not limited to, Type I hypersensitivity conditions (anaphylaxis and other basophil or mast cell mediated conditions), Type II hypersensitivity conditions (cytotoxic conditions involving phagocytosis or lysis of target cell), Type III hypersensitivity conditions (immune complex conditions involving antigen-antibody complexes), Type IV hypersensitivity conditions (cell-mediated conditions), transplant rejection, systemic lupus erythematosus, Sjogren's syndrome, CREST, scleroderma, polymyositis-dermatomyositis, mixed connective tissue disease, polyarteritis nodosa, amyloidosis, X-linked agammaglobulinemia, common variable immunodeficiency, isolated IgA deficiency, DiGeorge's syndrome, severe combined immunodeficiency, Wiscott-Aldrich syndrome, infection with HIV virus, acquired immune deficiency syndrome (AIDS), congenital anomalies of the immune system, hypersplenism, splenomegaly, congenital anomalies of the spleen, congestive splenomegaly, infarcts, or

splenic rupture.

Conditions, disorders, or diseases caused by a nutritional disease include, but are not limited to, marasmus, kwashiorkor, fat-soluble vitamin deficiency or toxicity (Vitamins A, D, E, or K), water-soluble vitamin deficiency or toxicity (thiamine, riboflavin, niacin, pyridoxine, folate, cobalamin, Vitamin C), mineral deficiency or toxicity (iron, calcium, magnesium, sodium, potassium, chloride, zinc, copper, iodine, cobalt, chromium, selenium, nickel, vanadium, manganese, molybdenum, rickets, osteomalacia, beriberi, hypoprothrombinemia, pellagra, megaloblastic anemia, scurvy, pernicious anemia, lack of gastric intrinsic factor, removal or pathophysiological functioning in the terminal ileum, microcytic anemia, or obesity.

Conditions, disorders, or diseases typically occurring in infancy or childhood include, but are not limited to, preterm birth, congenital malformations from genetic causes, congenital malformations from infectious causes, congenital malformations from toxic or teratogenic causes, congenital malformations from radiation, congenital malformations from idiopathic causes, small for gestational age infants, perinatal trauma, perinatal asphyxia, perinatal ischemia or hypoxia, birth injury, intracranial hemorrhage, deformations, respiratory distress syndrome of the newborn, atelectasis, hemolytic disease of the newborn, kernicterus, hydrops fetalis, congenital anemia of the newborn, icterus gravis, phenylketonuria, galactosemia, cystic fibrosis, hamartoma, or choristoma.

In another embodiment, the compounds and methods of the invention can be used to treat infections that cause cell death. The infections may be caused by bacteria; viruses; members of the family rickettsiae or chlamydia; fungi, yeast, hyphae or pseudohyphae; prions; protozoas; or metazoas.

Examples of aerobic or anaerobic bacteria which may cause such infections include, but are not limited to, gram-positive cocci, gram-positive bacilli (gram-positive rods), gram-negative cocci, gram-negative bacilli (gram-negative rods), Mycoplasma species, Ureaplasma species, Treponema species, Leptospira species, Borrelia species, Vibrio species, Mycobacteria species, members of Actinomycetes or L-forms (cell-wall deficient forms).

Examples of DNA, RNA or both DNA and RNA viruses which may cause such infections include, but are not limited to, members of the families adenoviridae, parvoviridae, papovaviridae, herpesviridae, poxviridae, picornaviridae, orthomyxoviridae, paramyxoviridae, rhabdoviridae, bunyaviridae, arenaviridae, coronaviridae, retroviridae, reoviridae, togaviridae and caliciviridae.

Examples of members of the families rickettsiae or chlamydiae which may cause such infections include, but are not limited to, Rickettsia species, Rochalimaea species, Coxiella species or Chlamydia species.

Examples of fungi, yeast, hyphae or pseudohyphae which may cause such infections include, but are not limited to, members of Ascomycota, Basidiomycota, Zygomycota, or Deutoeromycota (Fungi Imperfecti); Candida species, Cryptococcus species, Torulopsis species, Rhodotorula species, Sporothrix species, Phialophora species, Cladosporium species, Xylohypha species, Blastomyces species, Histoplasma species, Coccidioides species, Paracoccidioides species, Geotrichum species, Aspergillus species, Rhizopus species, Mucor species, Pseudoallescheria species or Absidia species.

Examples of prions which may cause such infections include, but are not limited to, the causative agent of Creutzfeldt-Jakob Disease, the causative agent of Gerstmann-Straussler-Scheinker Disease, the causative agent of fatal familial insomnia, the causative agent of kuru, and the causative agent of bovine spongiform encephalopathy.

Examples of protozoa at any point in their life cycle which may cause such infections include, but are not limited to, Entamoeba species, Naegleria species, Acanthamoeba species, Pneumocystis species, Balantidium species, members of order Leptomyxida, Plasmodium species, Toxoplasma species, Leishmania species and Trypanosoma species.

Examples of metazoa at any point in their life cycle which may cause such infections include, but are not limited to, members of Platyhelminthes such as the organisms in Cestoda (tapeworms) or Trematoda (flukes); or members of Aschelminthes such as the organisms in Acanthocephala, Chaetognatha, Cycliophora, Gastrotricha, Nematoda or Rotifera.

In a further embodiment, the compounds and methods of the invention can be used to treat infections or disorders which cause cell death in organ systems including, but not limited to, blood vessels, heart, red blood cells, white blood cells, lymph nodes, spleen, respiratory system, oral cavity, gastrointestinal tract, liver and biliary tract, pancreas, kidney, lower urinary tract, upper urinary tract and bladder, male sexual organs and genitalia, female sexual organs and genitalia, breast, thyroid gland, adrenal gland, parathyroid gland, skin, musculoskeletal system, bone marrow or bones.

In a further embodiment, the compounds and methods of the invention can be used to treat further physiological impacts on organs caused by the infections which induce

cell death including, but not limited to, fever equal to or greater than 101.5 degrees Fahrenheit, a decrease or increase in pulse rate by more than 20 beats per minute, a decrease or increase in supine systolic blood pressure by more than 30 millimeters of mercury, an increase or decrease in respiratory rate by more than 8 breaths per minute, an increase or decrease in blood pH by more than 0.10 pH units, an increase or decrease in one or more serum electrolytes outside of the clinical laboratory's usual reference range, an increase or decrease in the partial pressure of arterial oxygen or carbon dioxide outside of the clinical laboratory's usual reference range, an increase or decrease in white or red blood cells outside of the laboratory's usual reference range, an acute confusional state such as delirium where delirium is defined by the American Psychiatric Association's DSM-IV Manual or a diminished level of consciousness or attention.

5.4.1.2 Modulatory Antisense, Ribozyme and Triple Helix Approaches

In another embodiment, the types of conditions, disorders, or diseases involving cell death which may be prevented, delayed, or rescued by modulating protective sequence expression, protective sequence product activity, or their regulatory elements by using protective sequences in conjunction with well-known antisense, gene "knock-out," ribozyme and/or triple helix methods, are described. Among the compounds which may exhibit the ability to modulate the activity, expression or synthesis of the protective sequence, the protective sequence product, or its regulatory elements, including the ability to prevent, delay, or rescue a cell, cells, tissue, organ, or organism from the symptoms of a condition, disorder, or disease involving cell death are antisense, ribozyme and triple helix molecules. Such molecules may be designed to modulate, reduce or inhibit either unimpaired, or if appropriate, mutant protective sequence activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides which are complementary to a protective sequence mRNA. The antisense oligonucleotides will bind to the complementary protective

sequence mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required.

A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In one embodiment, oligonucleotides complementary to non-coding regions of the protective sequence of interest could be used in an antisense approach to inhibit translation of endogenous mRNA. Antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit protective sequence expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the cerebral RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleic acid of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended

groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger, *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, *BioTechniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier, *et al.*, 1987, *Nucl. Acids Res.* 15:6625-6641). The oligonucleotide is a 2'-0-

methylribonucleotide (Inoue, *et al.*, 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue, *et al.*, 1987, *FEBS Lett.* 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein, *et al.* (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin, *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), *etc.*

While antisense nucleotides complementary to the protective sequence coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

Antisense molecules should be delivered to cells that express the protective sequence *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies which specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

A preferred approach to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs which will form complementary base pairs with the endogenous protective sequence transcripts and thereby prevent translation of the protective sequence mRNA. For example, a vector can be introduced *e.g.*, such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3'-long terminal repeat

of Rous sarcoma virus (Yamamoto, *et al.*, 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, *Nature* 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct that can be introduced directly into the tissue site. Alternatively, viral vectors can be used that selectively infect the desired tissue, in which case administration may be accomplished by another route (*e.g.*, systemically).

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of target gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver, *et al.*, 1990, *Science* 247, 1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, *Current Biology* 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions which form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, *Nature*, 334:585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target gene mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena*

thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, *Science*, 224:574-578; Zaug and Cech, 1986, *Science*, 231:470-475; Zaug, *et al.*, 1986, *Nature*, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, *Cell*, 47:207-216). The Cech-type ribozymes have an eight base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences that are present in the target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, *etc.*) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies, *et al.*, 1985, *Nature* 317:230-234; Thomas and Capecchi, 1987, *Cell* 51:503-512; Thompson, *et al.*, 1989, *Cell* 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells which express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene

(*i.e.*, the target gene promoter and/or enhancers) to form triple helical structures which prevent transcription of the target gene in target cells in the body. (See generally, Helene, 1991, *Anticancer Drug Des.*, 6(6):569-584; Helene, *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 660:27-36; and Maher, 1992, *Bioassays* 14(12):807-815).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleic acids may be pyrimidine-based, which will result in TAT and CGC^+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen which are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles which the possibility may arise wherein the concentration of normal target gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of target gene activity are maintained, therefore, nucleic acid molecules which encode and express target gene polypeptides exhibiting normal target gene activity may, be introduced into cells via gene therapy methods such as those described, below, in Section 5.4.1.3 which do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the target gene encodes an

extracellular protein, it may be preferable to co-administer normal target gene protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid-phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.4.1.3 Gene Replacement Therapy

Protective nucleic acid sequences, described above in Section 5.1, can be utilized for transferring recombinant protective nucleic acid sequences to cells and expressing said sequences in recipient cells. Such techniques can be used, for example, in marking cells or for the treatment of a condition, disorder, or disease involving cell death. Such treatment can be in the form of gene replacement therapy. Specifically, one or more copies of a normal protective sequence or a portion of the protective sequence which directs the production of a protective sequence product exhibiting normal protective sequence function, may be inserted into the appropriate cells within a patient, using vectors which include, but are not limited to adenovirus, adeno-associated virus and retrovirus vectors, in addition to other particles which introduce DNA into cells, such as liposomes.

Because the protective sequence of the invention may be expressed in the brain, such gene replacement therapy techniques should be capable of delivering protective sequences to these cell types within patients. Thus, in one embodiment, techniques which are well known to those of skill in the art (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988) can be used to enable protective sequences to cross the blood-brain barrier readily and to deliver the sequences to cells in the brain. With respect to delivery which is capable of crossing the blood-brain barrier, viral vectors such as, for example, those described above, are preferable.

In another embodiment, techniques for delivery involve direct administration, *e.g.*, by stereotactic delivery of such protective sequences to the site of the cells in which the protective sequences are to be expressed.

Methods for introducing genes for expression in mammalian cells are well known in the field. Generally, for such gene therapy methods, the nucleic acid is directly administered *in vivo* into a target cell or a transgenic mouse that expresses SP-10 promoter operably linked to a reporter gene. This can be accomplished by any methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), by direct injection of naked DNA, by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), by coating with lipids or cell-surface receptors or transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, by administering it in linkage to a peptide which is known to enter the nucleus, or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993).

Additional methods which may be utilized to increase the overall level of protective sequence expression and/or gene product activity include using targeted homologous recombination methods, discussed in Section 5.2, above, to modify the expression characteristics of an endogenous protective sequence in a cell or microorganism by inserting a heterologous DNA regulatory element such that the inserted regulatory element is operatively linked with the endogenous protective sequence in question. Targeted homologous recombination can thus be used to activate transcription of an endogenous protective sequence which is "transcriptionally silent", *i.e.*, is not normally expressed or is normally expressed at very low levels, or to enhance the expression of an endogenous protective sequence which is normally expressed.

Further, the overall level of protective sequence expression and/or gene product activity may be increased by the introduction of appropriate protective sequence-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of a condition, disorder, or disease involving cell death. Such cells may be either recombinant or non-recombinant.

Among the cells that can be administered to increase the overall level of protective sequence expression in a patient are normal cells, preferably brain cells, which express the protective sequence. Alternatively, cells, preferably autologous cells, can be engineered to express protective sequences, and may then be introduced into a patient in positions appropriate for the amelioration of the symptoms of a condition, disorder, or disease involving cell death. Alternately, cells which express an unimpaired protective sequence and which are from a MHC matched individual can be utilized, and may include, for example, brain cells. The expression of the protective sequences is controlled by the appropriate gene regulatory sequences to allow such expression in the necessary cell types. Such gene regulatory sequences are well known to the skilled artisan. Such cell-based gene therapy techniques are well known to those skilled in the art, see, *e.g.*, Anderson, U.S. Patent No. 5,399,349.

When the cells to be administered are non-autologous cells, they can be administered using well-known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form that, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Additionally, compounds, such as those identified via techniques such as those described, in Section 5.4.2, which are capable of modulating protective sequences, protective sequence product activity, or their regulatory sequences can be administered using standard techniques which are well known to those of skill in the art. In instances in which the compounds to be administered are to involve an interaction with brain cells, the administration techniques should include well known methods that allow for a crossing of the blood-brain barrier.

5.4.1.4 Detection of Protective Nucleic Acid Molecules

A variety of methods can be employed to screen for the presence of protective sequence-specific mutations or polymorphisms (including polymorphisms flanking protective sequences) and to detect and/or assay levels of protective nucleic acid sequences.

Mutations or polymorphisms within or flanking the protective sequences can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures that are well known to those of skill in the art.

Protective nucleic acid sequences may be used in hybridization or amplification assays of biological samples to detect abnormalities involving protective sequence structure, including point mutations, insertions, deletions, inversions, translocations and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single-stranded conformational polymorphism analyses (SSCP) and PCR analyses.

Diagnostic methods for the detection of protective sequence-specific mutations or polymorphisms can involve for example, contacting and incubating nucleic acids obtained from a sample, *e.g.*, derived from a patient sample or other appropriate cellular source with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, such as described in Section 5.1, above, under conditions favorable for the specific annealing of these reagents to their complementary sequences within or flanking the protective sequence. The diagnostic methods of the present invention further encompass contacting and incubating nucleic acids for the detection of single nucleotide mutations or polymorphisms of the protective sequence. Preferably, these nucleic acid reagent sequences within the protective sequence are 15 to 30 nucleotides in length.

After incubation, all non-annealed nucleic acids are removed from the reaction. The presence of nucleic acids that have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described in Section 5.1 are easily removed. Detection of the remaining, annealed, labeled nucleic acid reagents is accomplished using standard techniques well known to those skilled in the art. The protective sequences of the invention to which the nucleic acid reagents have annealed can be

compared to the annealing pattern expected from a normal protective sequence of the invention in order to determine whether a protective sequence mutation is present.

In a preferred embodiment, protective sequence mutations or polymorphisms can be detected by using a microassay of nucleic acid sequences of the invention immobilized to a substrate or "gene chip" (see, *e.g.* Cronin, et al., 1996, Human Mutation 7:244-255). Alternative diagnostic methods for the detection of protective sequence-specific nucleic acid molecules (or flanking sequences), in patient samples or other appropriate cell sources, may involve their amplification, *e.g.*, by PCR (the experimental embodiment set forth in Mullis, 1987, U.S. Patent No. 4,683,202), followed by the analysis of the amplified molecules using techniques well known to those of skill in the art, such as, for example, those listed above. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of the protective sequence in order to determine whether a protective sequence mutation or polymorphism in linkage disequilibrium with a disease-causing allele exists.

Among those nucleic acid sequences that are preferred for such amplification-related diagnostic screening analyses are oligonucleotide primers that amplify exon sequences. The sequences of such oligonucleotide primers are, therefore, preferably derived from cerebral intron sequences so that the entire exon, or coding region, can be analyzed as discussed below. Primer pairs useful for amplification of cerebral exons are preferably derived from adjacent introns. Appropriate primer pairs can be chosen such that each of the cerebral exons present within the gene will be amplified. Primers for the amplification of exons can be routinely designed by one of ordinary skill.

Additional nucleic acid sequences which are preferred for such amplification-related analyses are those which will detect the presence of a polymorphism which differs from the sequence depicted in the Figures. Such polymorphisms include ones that represent mutations associated with a condition, disorder, or disease involving cell death.

Amplification techniques are well known to those of skill in the art and can routinely be utilized in connection with primers such as those described above. In general, hybridization conditions can be as follows: In general, for probes between 14 and 70 nucleotides in length, the melting temperature TM is calculated using the formula: Tm(°C)=81.5+16.6(log[monovalent cations])+0.41(% G+C)-(500/N) where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation Tm(°C)=81.5+16.6(log[monovalent

cations])+0.41(% G+C)-(0.61% formamide)-(500/N) where N is the length of the probe. Additionally, well-known genotyping techniques can be performed to identify individuals carrying protective sequence mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

Further, improved methods for analyzing DNA polymorphisms, which can be utilized for the identification of protective sequence-specific mutations, have been described which capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)n-(dG-dT)n short tandem repeats. The average separation of (dC-dA)n-(dG-dT)n blocks is estimated to be 30,000-60,000 bp. Markers which are so closely spaced exhibit a high frequency of co-inheritance, and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the protective sequence of the invention, and the diagnosis of diseases and disorders related to mutations of the protective sequences of the invention.

Also, Caskey *et al.* (U.S. Pat.No. 5,364,759) describe a DNA profiling assay for detecting short tri- and tetra nucleotide repeat sequences. The process includes extracting the DNA of interest, amplifying the extracted DNA and labeling the repeat sequences to form a genotypic map of the individual's DNA.

Other methods well known in the art may be used to identify single nucleotide polymorphisms (SNPs), including biallelic SNPs or biallelic markers which have two alleles, both of which are present at a fairly high frequency in a population. Conventional techniques for detecting SNPs include, *e.g.*, conventional dot blot analysis, single stranded conformational polymorphism (SSCP) analysis (see, *e.g.*, Orita *et al.*, 1989, *Proc. Natl. Acad. Sci. USA 86*:2766-2770), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection and other routine techniques well known in the art (see, *e.g.*, Sheffield *et al.*, 1989, *Proc. Natl. Acad. Sci. 86*:5855-5892; Grompe, 1993, *Nature Genetics 5*:111-117). Alternative, preferred methods of detecting and mapping SNPs involve microsequencing techniques wherein an SNP site in a target DNA is detecting by a single nucleotide primer extension reaction (see, *e.g.*, Goelet *et al.*, PCT Publication No. WO92/15712; Mundy, U.S. Patent No. 4,656,127; Vary and Diamond, U.S. Patent No. 4,851,331; Cohen *et al.*, PCT Publication No. WO91/02087; Chee *et al.*, PCT Publication

No. WO95/11995; Landegren *et al.*, 1988, *Science 241*:1077-1080; Nicerson *et al.*, 1990, *Proc. Natl. Acad. Sci. U.S.A. 87*:8923-8927; Pastinen *et al.*,1997, *Genome Res. 7*:606-614; Pastinen *et al.*, 1996, *Clin. Chem. 42*:1391-1397; Jalanko *et al.*, 1992, *Clin. Chem. 38*:39-43; Shumaker *et al.*, 1996, *Hum. Mutation 7*:346-354; Caskey *et al.*, PCT Publication No. WO 95/00669).

The level of protective sequence expression also can be assayed. For example, RNA from a cell type or tissue known, or suspected, to express the protective sequence, such as brain, may be isolated and tested utilizing hybridization or PCR techniques such as are described, above. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the protective sequence. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the protective sequence, including activation or inactivation of protective sequence expression.

In one embodiment of such a detection scheme, a cDNA molecule is synthesized from an RNA molecule of interest (*e.g.*, by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (*e.g.*, primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the protective sequence nucleic acid reagents described in Section 5.1. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform such protective sequence expression assays "*in situ*", *i.e.*, directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described in Section 5.1 may be used as probes and/or primers for such *in situ* procedures (see, for example, Nuovo, G.J., 1992, "PCR In Situ Hybridization: Protocols And Applications", Raven Press, NY).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern blot analysis can be performed to determine the level of mRNA expression of the protective sequence.

5.4.1.5 Detection of Protective Sequence Products

Protective sequence products of the invention, including both wild-type and mutant protective sequence products, conserved variants and polypeptide fragments thereof, which are discussed, above, in Section 5.2, may be detected using antibodies which are directed against such gene products. Such antibodies, which are discussed in Section 5.3, above, may thereby be used as diagnostics and prognostics for a condition, disorder, or disease involving cell death. Such methods may be used to detect abnormalities in the level of protective sequence expression or of protective sequence product synthesis, or abnormalities in the structure, temporal expression and/or physical location of protective sequence product. The antibodies and immunoassay methods described herein have, for example, important *in vitro* applications in assessing the efficacy of treatments for conditions, disorders, or diseases involving cell death. Antibodies, or fragments of antibodies, such as those described below, may be used to screen potentially therapeutic compounds *in vitro* to determine their effects on protective sequence expression and protective sequence product production. The compounds which have beneficial effects on conditions, disorders, or diseases involving cell death can thereby be identified, and a therapeutically effective dose determined.

In vitro immunoassays may also be used, for example, to assess the efficacy of cell-based gene therapy for a condition, disorder, or disease involving cell death. Antibodies directed against protective sequence products may be used *in vitro* to determine, for example, the level of protective sequence expression achieved in cells genetically engineered to produce the protective sequence product. In the case of intracellular protective sequence products, such an assessment is done, preferably, using cell lysates or extracts. Such analysis will allow for a determination of the number of transformed cells necessary to achieve therapeutic efficacy *in vivo*, as well as optimization of the gene replacement protocol.

The tissue or cell type to be analyzed generally will include those that are known, or suspected, to express the protective sequence. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, New York). The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the protective sequence.

Preferred diagnostic methods for the detection of protective sequence products, conserved variants or peptide fragments thereof, may involve, for example, immunoassays wherein the protective sequence products or conserved variants or peptide fragments are detected by their interaction with an anti-protective sequence product-specific antibody.

For example, antibodies, or fragments of antibodies, such as those described, above, in Section 5.3, may be used to, quantitatively or qualitatively, detect the presence of protective sequence products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below, this Section) coupled with light microscopic, flow cytometric or fluorimetric detection. Such techniques are especially preferred for protective sequence products that are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of protective sequence products, conserved variants or peptide fragments thereof. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody which binds to a protective sequence polypeptide. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the protective sequence product, conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily recognize that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve *in situ* detection of a protective sequence product.

Immunoassays for protective sequence products, conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells or lysates of cells in the presence of a detectably labeled antibody capable of identifying the protective sequence product, conserved variants or peptide

fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier, such as nitrocellulose, which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled protective sequence product specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One of the ways in which the protective sequence product-specific antibody can be detectably labeled is by linking the same to an enzyme, such as for use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, A. *et al.*, 1978, *J. Clin. Pathol.* 31:507-520; Butler, J.E., 1981, *Meth. Enzymol.* 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, FL,; Ishikawa, E. *et al.*, (eds.), 1981, Enzyme Immunoassay, Kgaku Shoin, Tokyo). The enzyme that is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol

dehydrogenase, α-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods that employ a chromogenic substrate for the enzyme. Detection also may be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished also using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect protective sequence products through the use of a radioimmunoassay (RIA) (*see*, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of

luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

5.4.2 Screening Assays for Compounds which Interact with Protective Sequence Products or Modulate Protective Sequence Activity

The following assays are designed to identify compounds which bind to a protective sequence product, compounds which bind to proteins, or portions of proteins which interact with a protective sequence product, compounds which modulate, *e.g.*, interfere with, the interaction of a protective sequence product with proteins and compounds which modulate the activity of the protective sequence (*i.e.*, modulate the level of protective sequence expression and/or modulate the level of protective sequence product activity). Assays may additionally be utilized which identify compounds which bind to protective sequence regulatory sequences (*e.g.*, promoter sequences; see *e.g.*, Platt, 1994, J. Biol. Chem. 269, 28558-28562), and which can modulate the level of protective sequence expression. Such compounds may include, but are not limited to, small organic molecules, such as ones which are able to cross the blood-brain barrier, gain to and/or entry into an appropriate cell and affect expression of the protective sequence or some other gene involved in a protective sequence regulatory pathway.

Methods for the identification of such proteins are described, below, in Section 5.4.2.2. Such proteins may be involved in the control and/or regulation of functions related to cell death. Further, among these compounds are compounds which affect the level of protective sequence expression and/or protective sequence product activity and which can be used in the therapeutic treatment of conditions, disorders, or diseases involving cell death as described, below, in Section 5.4.2.3.

Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to, Ig-tailed fusion peptides, and members of random peptide libraries; (see, *e.g.*, Lam, *et al.*, 1991, *Nature* 354:82-84; Houghten, *et al.*, 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, *et al.*, 1993, *Cell* 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or

inorganic molecules.

Such compounds may further comprise compounds, in particular drugs or members of classes or families of drugs, known to ameliorate the symptoms of a condition, disorder, or disease involving cell death.

Such compounds include families of antidepressants such as lithium salts, carbamazepine, valproic acid, lysergic acid diethylamide (LSD), *p*-chlorophenylalanine, *p*-propyldopacetamide dithiocarbamate derivatives *e.g.*, FLA 63; anti-anxiety drugs, *e.g.*, diazepam; monoamine oxidase (MAO) inhibitors, *e.g.*, iproniazid, clorgyline, phenelzine and isocarboxazid; biogenic amine uptake blockers, *e.g.*, tricyclic antidepressants such as desipramine, imipramine and amitriptyline; serotonin reuptake inhibitors *e.g.*, fluoxetine; antipsychotic drugs such as phenothiazine derivatives (*e.g.*, chlorpromazine (thorazine) and trifluopromazine)), butyrophenones (*e.g.*, haloperidol (Haldol)), thioxanthene derivatives (*e.g.*, chlorprothixene), and dibenzodiazepines (*e.g.*, clozapine); benzodiazepines; dopaminergic agonists and antagonists *e.g.*, L-DOPA, cocaine, amphetamine, α-methyl-tyrosine, reserpine, tetrabenazine, benzotropine, pargyline; noradrenergic agonists and antagonists *e.g.*, clonidine, phenoxybenzamine, phentolamine, tropolone.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of protective sequence products and for ameliorating conditions, disorders, or diseases involving cell death. Assays for testing the effectiveness of compounds identified by, for example, techniques such as those described in Sections 5.4.2.1 - 5.4.2.3, are discussed, below, in Section 5.4.2.4.

5.4.2.1 *In Vitro* Screening Assays for Compounds which Bind to Protective Sequence Products

In vitro systems may be designed to identify compounds capable of binding the protective sequence products of the invention. Compounds identified may be useful, for example, in modulating the activity of unimpaired and/or mutant protective sequence products, may be useful in elaborating the biological function of the protective sequence product, may be utilized in screens for identifying compounds which disrupt normal protective sequence product interactions or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds which bind to the protective sequence product involves preparing a reaction mixture of the protective sequence

product and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay involves anchoring a protective sequence product or a test substance onto a solid support and detecting protective sequence product/test compound complexes formed on the solid support at the end of the reaction. In one embodiment of such a method, the protective sequence product may be anchored onto a solid support, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates are conveniently utilized as the solid support. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the previously non-immobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for the protective sequence product or the test compound to

anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

5.4.2.2 Assays for Proteins which Interact with Protective Sequence Products

Any method suitable for detecting protein-protein interactions may be employed for identifying protective sequence product-protein interactions.

Among the traditional methods that may be employed are co-immunoprecipitation, cross-linking and co-purification through gradients or chromatographic columns. Utilizing procedures such as these allows for the identification of proteins, including intracellular proteins, which interact with protective sequence products. Once isolated, such a protein can be identified and can be used in conjunction with standard techniques, to identify proteins it interacts with. For example, at least a portion of the amino acid sequence of a protein which interacts with the protective sequence product can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, *e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles," W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the screening are well known. (See, *e.g.*, Ausubel, *supra*, and 1990, "PCR Protocols: A Guide to Methods and Applications," Innis, *et al.*, eds. Academic Press, Inc., New York).

Additionally, methods may be employed which result in the simultaneous identification of genes that encode a protein that interacts with a protective sequence product. These methods include, for example, probing expression libraries with labeled protective sequence product, using the protective sequence product in a manner similar to the well-known technique of antibody probing of λgt11 libraries.

One method that detects protein interactions *in vivo*, the two-hybrid system, is described in detail for illustration only and not by way of limitation. One version of this

system has been described (Chien, *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed which encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to the protective sequence product and the other consists of the transcription activator protein's activation domain fused to an unknown protein which is encoded by a cDNA which has been recombined into this plasmid as part of a cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (*e.g.*, HBS or *lacZ*) whose regulatory region contains the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodologies may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, protective sequence products of the invention may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a bait protective sequence product fused to the DNA-binding domain are co-transformed into a yeast reporter strain, and the resulting transformants are screened for those which express the reporter gene. For example, a bait protective sequence, such as the open reading frame of the gene, can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line, from which proteins which interact with bait protective sequence products are to be detected, can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the

cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. Such a library can be co-transformed along with the bait protective sequence-GAL4 fusion plasmid into a yeast strain that contains a lacZ gene driven by a promoter that contains GAL4 activation sequence. A cDNA encoded protein, fused to a GAL4 transcriptional activation domain that interacts with bait protective sequence product will reconstitute an active GAL4 protein and thereby drive expression of the HIS3 gene. Colonies that express HIS3 can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the bait protective sequence product-interacting protein using techniques routinely practiced in the art.

5.4.2.3 Assays for Compounds which Interfere with or Potentiate Protective Sequence Products Macromolecule Interaction

The protective sequence products may, *in vivo*, interact with one or more macromolecules, including intracellular macromolecules, such as proteins. Such macromolecules may include, but are not limited to, nucleic acid molecules and those proteins identified via methods such as those described, above, in Sections 5.4.2.1 - 5.4.2.2. For purposes of this discussion, the macromolecules are referred to herein as "binding partners". Compounds that disrupt protective sequence product binding to a binding partner may be useful in regulating the activity of the protective sequence product, especially mutant protective sequence products. Such compounds may include, but are not limited to molecules such as peptides, and the like, as described, for example, in Section 5.4.2.1 above.

The basic principle of an assay system used to identify compounds which interfere with or potentiate the interaction between the protective sequence product and a binding partner or partners involves preparing a reaction mixture containing the protective sequence product and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of protective sequence product and its binding

partner. Control reaction mixtures are incubated without the test compound or with a compound that is known not to block complex formation. The formation of any complexes between the protective sequence product and the binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the protective sequence product and the binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal protective sequence product also may be compared to complex formation within reaction mixtures containing the test compound and a mutant protective sequence product. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal protective sequence product.

In order to test a compound for potentiating activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of protective sequence product and its binding partner. Control reaction mixtures are incubated without the test compound or with a compound that is known not to block complex formation. The formation of any complexes between the protective sequence product and the binding partner is then detected. Increased formation of a complex in the reaction mixture containing the test compound, but not in the control reaction, indicates that the compound enhances and therefore potentiates the interaction of the protective sequence product and the binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal protective sequence product may also be compared to complex formation within reaction mixtures containing the test compound and a mutant protective sequence product. This comparison may be important in those cases wherein it is desirable to identify compounds that enhance interactions of mutant but not normal protective sequence product.

In alternative embodiments, the above assays may be performed using a reaction mixture containing the protective sequence product, a binding partner and a third compound which disrupts or enhances protective sequence product binding to the binding partner. The reaction mixture is prepared and incubated in the presence and absence of the

test compound, as described above, and the formation of any complexes between the protective sequence product and the binding partner is detected. In this embodiment, the formation of a complex in the reaction mixture containing the test compound, but not in the control reaction, indicates that the test compound interferes with the ability of the second compound to disrupt protective sequence product binding to its binding partner.

The assays for compounds that interfere with or potentiate the interaction of the protective sequence products and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the protective sequence product or the binding partner onto a solid support and detecting complexes formed on the solid support at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds which interfere with or potentiate the interaction between the protective sequence products and the binding partners, *e.g.*, by competition, can be identified by conducting the reaction in the presence of the test substance; *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the protective sequence product and interactive intracellular binding partner. Alternatively, test compounds which disrupt preformed complexes, *e.g.*, compounds with higher binding constants which displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the protective sequence product or the interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protective sequence product or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is

complete, unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex formation or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the protective sequence product and the interactive binding partner is prepared in which either the protective sequence product or its binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, *e.g.*, U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt protective sequence product/binding partner interaction can be identified.

In another embodiment of the invention, these same techniques can be employed using peptide fragments which correspond to the binding domains of the protective sequence product and/or the binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely

practiced in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this Section above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the segments is engineered to express peptide fragments of the protein, it can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, a protective sequence product can be anchored to a solid material as described, above, in this Section by making a GST-1 fusion protein and allowing it to bind to glutathione agarose beads. The binding partner can be labeled with a radioactive isotope, such as ^{35}S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-1 fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the binding partner binding domain, can be eluted, purified and analyzed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or produced using recombinant DNA technology.

5.4.2.4 Assays for the Identification of Compounds which Modulate Conditions, Disorders, or Diseases Involving Cell Death

Compounds, including, but not limited to, binding compounds identified via assay techniques such as those described, above, in Sections 5.4.2.1 - 5.4.2.3, can be tested for the ability to ameliorate symptoms of a condition, disorder, or disease involving cell death.

It should be noted that the assays described herein can be used to identify compounds which affect activity by either affecting protective sequence expression or by affecting the level of protective sequence product activity. For example, compounds may be identified which are involved in another step in the pathway in which the protective sequence and/or protective sequence product is involved, such as, for example, a step which is either "upstream" or "downstream" of the step in the pathway mediated by the protective sequence. Such compounds may, by affecting this same pathway, modulate the effect on the development of conditions, disorders, or diseases involving cell death. Such compounds can be used as part of a therapeutic method for the treatment of the condition, disorder, or disease.

Described below are cell-based and animal model-based assays for the identification of compounds exhibiting such an ability to ameliorate symptoms of a condition, disorder, or disease involving cell death.

First, cell-based systems can be used to identify compounds which may act to ameliorate symptoms of a condition, disorder, or disease, including, but not limited to, those described in Section 5.4.1.1. Such cell systems can include, for example, recombinant or non-recombinant cell, such as cell lines, which express the protective sequence of interest.

In utilizing such cell systems, cells which express the protective sequence of interest may be exposed to a compound suspected of exhibiting an ability to ameliorate symptoms of a condition, disorder, or disease involving cell death at a sufficient concentration and for a sufficient time to elicit such an amelioration of such symptoms in the exposed cells. After exposure, the cells can be assayed to measure alterations in the expression of the protective sequence, *e.g.*, by assaying cell lysates for cerebral mRNA transcripts (*e.g.*, by Northern analysis) or for protective sequence products expressed by the cell; compounds which modulate expression of the protective sequence are good candidates as therapeutics.

In addition, animal-based systems or models for a condition, disorder, or disease involving cell death, for example, transgenic mice containing a human or altered form of a protective sequence, may be used to identify compounds capable of ameliorating symptoms of the condition, disorder, or disease. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions. For

example, animal models may be exposed to a compound suspected of exhibiting an ability to ameliorate symptoms, at a sufficient concentration and for a sufficient time to elicit such an amelioration of symptoms of a condition, disorder, or disease involving cell death. The response of the animals to the exposure may be monitored by assessing the reversal of the symptoms of the condition, disorder, or disease.

With regard to intervention, any treatments that reverse any aspect of symptoms of a condition, disorder, or disease involving cell death, should be considered as candidates for human therapeutic intervention in such conditions, disorders, or diseases. Dosages of test agents may be determined by deriving dose-response curves, as discussed in Section 5.5.1, below.

5.4.3 Additional Uses for the Protective Sequences, Protective Sequence Products, or Their Regulatory Elements

In addition to the uses described above, the polynucleotides of the present invention can be used for various other purposes. For example, they can be used to express recombinant protein for analysis, characterization or therapeutic use; as molecular weight markers on gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic conditions, disorders, or diseases; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response.

The proteins provided by the present invention can similarly be used to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands.

Where the protein binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

5.5 Pharmaceutical Preparations and Methods of Administration

The compounds which are determined to affect protective sequence expression or gene product activity can be administered to a patient at therapeutically effective doses to treat or ameliorate a condition, disorder, or disease involving cell death or modulate a cell death-related process described herein. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of such a condition, disorder, or disease.

5.5.1 Effective Dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds which exhibit toxic side effects may be used, care should be taken to design a delivery system which targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range which includes the IC_{50} (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of antibody, protein, or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or condition, disorder, or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

5.5.2 Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral rectal or topical administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*,

dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In certain embodiments, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

For topical application, the compounds may be combined with a carrier so that an effective dosage is delivered, based on the desired activity.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange

resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

6 EXAMPLE: SEQUENCE AND CHARACTERIZATION OF PROTECTIVE SEQUENCES

In the example presented herein, the sequence and characterization of the protective sequences are provided.

6.1 Materials and Methods

6.1.1 Preparation of DNA

A human fetal brain cDNA library (Gibco), in which individual clones were inserted into the NotI-SalI site of the pCMV•SPORT2 vector, was diluted 200,000 fold in LB broth (DIFCO Laboratories) containing 0.2 mg/ml ampicillin (Sigma). The diluted library (100-140 µl) was then plated and grown on LB agar (DIFCO Laboratories) bioassay plates with 0.2 mg/ml ampicillin. Plates were incubated at 37°C for 24 hours. Single colonies were then used to inoculate deep-well blocks containing 1.5 ml LB broth containing 0.2 mg/ml ampicillin. Inoculated cultures were incubated at 37°C with agitation at 150-200 rpm for 18-24 hours. Replicate plates were created from the cultures by adding 20 µl of culture to 80 µl of LB broth containing 18% glycerol and 0.2 mg/ml ampicillin and stored at -80°C. Remaining bacterial cells were centrifuged at 1000 x g for 6 minutes to collect the cells at the bottom. Following centrifugation, the broth was decanted off of the bacterial pellet and the pellet resuspended and then stored in 100 µl of Cell Resuspension Solution (Promega) at 4°C for up to one week.

Plasmid DNA was extracted using Promega MagneSil kits with a modified protocol. The pelleted bacteria were re-suspended and 50 µl was transferred into a round bottom plate that rests on a magnet. Cell Lysis Solution (50 µl) was added and the plate was incubated at room temperature without agitation for 30 seconds. Following lysis, 70 µl of a Neutralization Solution/MagneSil Paramagnetic Particles mixture (pre-mixed at a ratio of

6:1) was added. The reaction was mixed by pipetting and incubated at room temperature without agitation for 5 minutes to allow the magnetic particles to be drawn to the magnet. The supernatant containing plasmid DNA was then transferred to a new plate and stored at -20°C.

Individual clones were chosen for their ability to delay or prevent cell death when introduced into a cell predisposed to undergoing cell death, relative to a corresponding cell into which no exogenous protective sequence had been introduced.

6.1.2 Sequence Characterization of the DNA

The cDNA inserts of the clonally pure plasmids which are selected for their ability to protect cells from cell death when introduced into cells predisposed to undergo cell death are sequenced using the ABI Big Dye terminator Cycle Sequencing Ready Reaction Kit and subsequently analyzed on the ABI310 capillary sequencing machine (PE Biosystems, Foster City, CA).

Briefly, 0.5 μg of plasmid DNA is mixed with 3.2 pmole of either the M13 forward (5'-TGTAAAACGACGGCCAGT-3'; SEQ ID NO:465) or the M13 reverse (5'-CAGGAAACAGCTATGACC-3'; SEQ ID NO:466) sequencing primer and 8 μl of the terminator ready reaction mix in a total volume of 20 μl. The cycle sequencing reaction is carried out in a thermocycler (PCR machine) using standard methods known by those skilled in the art. The extension products from the sequencing reaction are purified by precipitation using isopropanol. 80 μl of 75% isopropanol is added to the sample and after thorough mixing, the sample is incubated at room temperature (25°C) for 20 minutes. The sample is then centrifuged at 12,000 x g for 20 minutes at room temperature. The supernatant is removed and the pellet is rinsed once by addition of 250 μl of 75% isopropanol followed by centrifugation as above for 5 minutes. The supernatant is removed and the sample air-dried for 10 minutes. The sample is then resuspended in 20 μl of TSR (template suppression reagent) and denatured by heating at 94°C for 2 minutes and rapidly cooling on ice. The subsequent electrophoresis and analysis is carried out on the ABI310 sequencer according to the manufacturer's protocol. The entire cDNA clone is similarly sequenced by the use of sequence specific internal primers as required.

6.1.3 Sequence Comparison

The sequence data for the protective cDNA clones is compared using the BLAST 2.0 algorithm (Altschul, SF *et al.*, 1997, Nuc. Acids Res. 25:3389) against known sequences in the GeneBank sequence database maintained by NCBI (National Center for Biotechnology Information). This program uses the two-hit method to find homology within the database. The BLAST nucleotide searches are performed with the "BLAST N" program (wordlength = 11) to obtain nucleic acids homologous to nucleic acid molecules of the invention. BLAST protein searches of potential ORFs are performed with the "BLAST P" program (wordlength = 3) to obtain amino acid sequences homologous to the ORFs of the invention.

6.1.4 Immuno-Cytochemistry Protocol for the Characterization of Protected Cells

Transfected tissue is immersed in freshly prepared 2.5% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for two hours to fix the tissue. PFA is removed by aspiration and the fixed tissue washed consecutively four times in PBS for 15 minutes, changing the PBS solution between each wash. Upon removal of the final PBS wash, the tissue is immersed in a blocking solution consisting of 10% goat serum, 2% bovine serum albumin (BSA), and 0.25% Triton X-100 for a duration of two hours.

After removal of the blocking solution, the tissue is immersed in a primary antibody solution, freshly prepared by adding rabbit anti-GFP polyclonal (1:2000 ul) into blocking solution, for an incubation period of twelve hours at 4°C.

After removal of the primary antibody solution, the tissue is washed consecutively four times in PBS for 10 minutes, changing the PBS solution between each wash. An anti-rabbit, flourescently conjugated secondary antibody, diluted in PBS at a concentration of 1:500, is then added to the tissue and allowed to incubate at room temperature for four hours. The secondary antibody solution is removed by aspiration and the tissue washed consecutively four times in PBS for 15 minutes, changing the PBS solution between each wash. After the final wash is removed, the tissue is mounted on glass slides and dried at 37°C for thirty minutes. A three-minute xylene incubation is performed before the addition of coverslips to preserve the slices.

6.2 Results

The following protective sequences, which were obtained using the methods

described in Section 6.1, were chosen based on their ability to prevent, delay, or rescue cells predisposed to undergo cell death, relative to a corresponding cell into which no exogenous protective sequence had been introduced.

6.2.1 Protective sequence CNI-00711

Protective sequence CNI-00711 (SEQ ID NO:1) comprises 852 nucleotides. Twelve (12) potential open reading frames ("ORFs") have been identified within the protective sequence and are depicted in Table 2. BLAST sequence comparison analysis of CNI-00711 against known sequences in the GenBank sequence database reveals 89% homology, at the nucleotide level, with the UV1 exon, containing part of the envelope region of a human endogenous retrovirus (HERV) type C (ACC. No. AF058907). The homologous clone was initially described as a germ-line insertion of HERV into the human pleiotrophin gene. The insertion occurred between the 5' untranslated region (UTR) and the coding region. The homology between CNI-00711 and HERV exists for 366 base pairs out of the 412 bases found within the UV1 exon of the HERV insertion.

6.2.2 Protective sequence CNI-00712

Protective sequence CNI-00712 (SEQ.ID NO:26) is a completely novel sequence which comprises 1096 nucleotides. Twenty-four (24) potential ORFs have been identified within the protective sequence and are depicted in Table 3. The longest ORF of the clone is 160 amino acids. BLAST sequence comparison analysis of CI-00712 against known nucleotide and protein sequences in the GenBank database reveals no significant homology at either the nucleotide or amino acid level.

6.2.3 Protective sequence CNI-00714

Protective sequence CNI-00714 (SEQ. ID NO:75) comprises 1825 nucleotides. Thirty (30) potential ORFs have been identified within the protective sequence and are depicted in Table 4. The longest ORF of the cDNA encodes 412 amino acids. BLAST sequence comparison analysis of CNI-00714 against known nucleic acids in the GenBank database reveals homology with the sequence encoding the human KIAA0764 gene

(ACC. No. AB018307). At the nucleotide level, the overall percent homology between CNI-00714 and KIAA0764 is 76%. At the amino acid level, the CNI-00714 and KIAA0764 proteins are identical except for a 2 amino acid deletion near the N-terminus. KIAA0764 is an unidentified brain cDNA that shows high level expression in lung and brain.

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6.2.4 Protective sequence CNI-00715

Protective sequence CNI-00715 (SEQ. ID NO:136) comprises 542 nucleotides. Eight (8) potential ORFs have been identified within the protective sequence and are depicted in Table 5. BLAST sequence comparison analysis of CNI-00715 against known nucleic acids in the GenBank database reveals a 97% identity (503/520 bases) with a human DNA sequence, clone 425C14, which is from chromosome 6Q22. This clone contains the heat shock factor 2 gene (HSF2) and an unknown gene which is similar to the gene which encodes the placental protein, DIFF33 (ACC. No. HS425C14). Additionally, a relatively high homology – 74% identity (64/87 bases) – is also observed with a short region within the coding region of the bestrophin gene. Bestrophin is the gene responsible for Best macular dystrophy (ACC.No. AF057170).

6.2.5 Protective sequence CNI-00716

Protective sequence CNI-00716 (SEQ. ID NO:153) is a completely novel sequence which comprises 771 nucleotides. Fifteen (15) potential ORFs have been identified within the protective sequence and are depicted in Table 6. The longest ORF is 58 amino acids. BLAST sequence comparison analysis of CNI-00716 against known nucleotide and protein sequences in the GenBank database reveals no significant homology at either the nucleotide or the amino acid level.

25

6.2.6 Protective sequence CNI-00717

Protective sequence CNI-00717 (SEQ ID NO:184) comprises 1669 nucleotides. Thirty eight (38) potential ORFs have been identified within the protective sequence and are depicted in Table 7. BLAST sequence comparison analysis of CNI-00717 against known nucleic acids in the GenBank database reveals 61% (573/935 bases) identity

within the coding region of the mouse GARP34 mRNA (ACC No. AB018374). The ORF of CNI-00717 in this region of homology is 272 amino acids in length. When this amino acid sequence is compared to the amino acid sequence of GARP34, there is 50% identity (132/265 amino acids).

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6.2.7 Protective sequence CNI-00720

Protective sequence CNI-00720 (SEQ. ID NO:261) comprises 1182 nucleotides. Fifteen (15) potential ORFs have been identified within the protective sequence and are depicted in Table 8. Two relatively long ORFs of 75 and 89 amino acids were documented. Neither ORF was homologous to any known sequences in the Genbank database. BLAST sequence comparison analysis of CNI-00720 against known nucleic acids in the GenBank database reveals homology with the 3'UTR of two human genes – neuroendocrine-specific protein-like protein 1(NSPL1) (ACC.No. 119297) and reticulon 3 (RTN3) (ACC. No. RTN3). NSPL1 and RTN 3 are identical neuron-specific genes that belong to the reticulon gene family. There is 99% identity between the nucleic acid of CNI-00720 and the 3' UTRs of NSPL1 and RTN3. Additionally, there is 97% identity within the 5' UTR of the human protein tyrosine kinase, t-Ror-1(Ror1) mRNA (ACC. No.HSU38894)..

6.2.8 Protective sequence CNI-00721

Protective sequence CNI-00721 (SEQ. ID NO:292) comprises 1965 nucleotides. Thirty-three (33) potential ORFs have been identified within the protective sequence and are depicted in Table 9. BLAST sequence comparison analysis of CNI-00721 against known nucleic acids in the GenBank database revealed strong homology with the human p311 mRNA (ACC. No.HSU36189). There is 99% identity (465/470 bases) between the CNI-00721 cDNA and the p311 mRNA. The homology is 100% within the coding sequence region.

6.2.9 Protective sequence CNI-00723

Protective sequence CNI-00723 (SEQ. ID NO:359) comprises 2702 nucleotides. Fifty-one (51) potential ORFs have been identified within the protective

sequence and are depicted in Table 10. BLAST sequence comparison analysis of CNI-00723 against known nucleic acids in the GenBank database reveals a short stretch of homology with the Drosophila asteroid mRNA (ACC.No AF047010). The CNI-00723 cDNA is 35% identical (73/209 bases) to the asteroid mRNA with the homology occurring within the coding region of the mRNA. The longest ORF of CNI-00723 is 490 amino acids. This ORF is 37% identical (55/144 amino acids) to the Drosophila asteroid protein.

6.2.10 Protective sequence CNI-00724

Protective sequence CNI-00724 (SEQ. ID NO:462) is a completely novel sequence which comprises 979 nucleotides. Only a single potential ORF has been identified within the protective sequence and it is depicted in Table 11. This ORF, which is 80 amino acids in length, revealed no homology with any known amino acid sequence in the GenBank database. BLAST sequence comparison analysis of CNI-00724 against known nucleic acids in the GenBank database reveals that homology exists with a human VGF nerve growth factor inducible mRNA (ACC. No. NM_003378). There is 94% identity (821/872 bases) between the CNI-00724 insert and the VGF nerve growth factory inducible mRNA. This homology is observed in the 3' portion of the coding sequence and in the 3' UTR of the VGF nerve growth factory inducible mRNA.

7 DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with the Agricultural Research Service (NRRL), U. S. Department of Agriculture, 1815 N. University Street, Peoria, Illinois, 61604, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and comply with the criteria set forth in 37 C.F.R. § 1.801-1.809 regarding availability and permanency of deposits. The deposits were made on the date indicated and assigned the indicated accession number:

Microorganism Deposit	NRRL Deposit No.	Date of Deposit
Escherichia coli CNI-NPP1-CP10	B-30231	November 3, 1999

CNI-NPP1-CP10 represents a composite deposit of a mixture of ten (10) strains. To distinguish and isolate each of the individual strains, an aliquot of the mixture can be streaked out to single colonies on nutrient media (*e.g.*, LB plates) supplemented with 100μg/ml ampicillin, single colonies grown and then DNA can be extracted using standard procedures.

Next, a sample of the DNA preparation can be digested with *Not* I and *Sal* I, and the resulting products can be separated by standard gel electrophoresis techniques using a 1% agarose gel in TAE buffer. Liberated inserts are of the following approximate sizes:

1:	CNI-00711	852 bp
2:	CNI-00712	1096 bp
3:	CNI-00714	1825 bp
4:	CNI-00715	542 bp
5:	CNI-00716	771 bp
6:	CNI-00717	1669 bp
7:	CNI-00720	1182 bp
8:	CNI-00721	1965 bp
9:	CNI-00723	2702 bp
10:	CNI-00724	979 bp

8 REFERENCES CITED

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.